The antimicrobial defence system of avian egg albumen.

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THE ANTIMICROBIAL DEFENCE SYSTEM OF AVIAN EGG ALBUMEN

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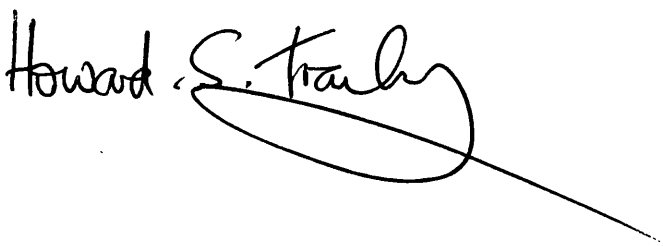

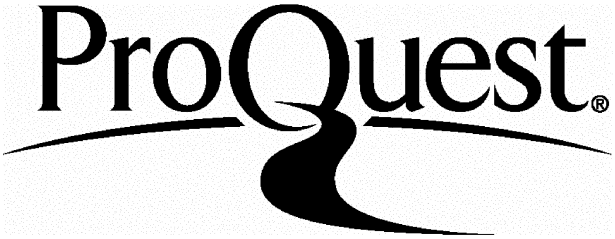

To my father and mother, Harold and Millicent Tranter

and especially

For my wife Susan Anne

I think that if required on pain of death to name
instantly the most perfect thing in the universe,
I should risk my fate upon a bird's egg -

T.W. Higginson.

CONTENTS

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ABSTRACT

The components of avian eggs have attracted the attention of workers from all scientific fields but since the early descriptive investigations most studies have been concerned with the fundamental problems of protein chemistry, molecular biology, genetics etc., rather than the overall contributions of these proteins to the well being of the embryo. There is a large scattered literature on the occurrence, chemical and biological properties and phylogenetic traits of egg white proteins; this study brings together much of this literature and deals with the physico-chemical properties of these proteins with respect to their antimicrobial nature. It also presents a brief look at the consequences of iron deprivation in microbial metabolism, a situation that occurs through the metal-chelating properties of albumen ovotransferrin.

Studies were made on the antimicrobial mechanism of avian egg whites, with particular reference to hen egg albumen, and the major contributory factors identified. The respective influences of the two major biological proteins of egg albumen, lysozyme and ovotransferrin, were assessed using a wide range of micro-organisms including bacteria and yeast vegetative cells and bacterial endospores. In the case of bacteria, two mechanisms of inhibition by hen egg albumen, bacteriostasis and a bactericidal activity, were observed. Although iron deprivation was shown to be directly responsible for both of these mechanisms, a new interpretation of the importance of high alkaline pH in preventing microbial scavenging of this element is proposed. The importance of incubation temperature and pH on these mechanisms of inhibition were demonstrated.

The antimicrobial system of avian egg albumen is discussed in

relation to equivalent systems that occur in mammalian serum and milk. Finally a possible practical application of the antimicrobial mechanisms of avian egg albumen was demonstrated by inhibition of microbial growth in reconstituted dried baby feed and the relevance of this type of system in developing countries discussed.

INTRODUCTION

The breeding success of birds is dependent in part upon the development of an embryo in a cleidoic (closed-box) egg (Needham, 1931). As it contains all the nutrients (except for oxygen) and water required for prenatal development and the maintenance of the chick for a few hours or days after hatching, the egg can be regarded as a large investment of energy on the part of the female. The scale of this investment is considered to have had a profound selective influence on factors such as clutch size, breeding periods etc. (Lack, 1968). Given that there is a limited amount of energy available for reproduction, this must be allocated optimally to eggs, avoidance of predation and competitive ability. The vulnerability of the egg and also the parents to predation has been appreciated but undue emphasis has been given to "macro-predators" and little attention to the mechanisms that protect the major food reserve, the yolk, and the embryo from infection and breakdown by micro-organisms.

Although the egg is self-contained with respect to water and nutrients the embryo's requirement for exchange of respiratory gases requires a porous shell. As will be discussed subsequently, these pores can allow micro-organisms to enter the egg and infect the contents. Thus a priori there would appear to be a need for a defence system that protects eggs in the event of translocation of micro-organisms across the shell. The literature contains many references to the antimicrobial factors present in egg albumen, particularly that of domestic hens, but only a tentative description of the albumen's defence potential is possible because few studies have been concerned with the whole albumen and even fewer with the influence of temperature on its functioning. The following literature review summarizes the relevant information on the microbiology of

eggs, discusses in detail the physico-chemical properties of the albumen proteins that could contribute to the egg's antimicrobial defence and describes the means whereby micro-organisms acquire iron when this element is in low concentration.

In the present study, the major emphasis was given to the behaviour of selected micro-organisms in albumen incubated under various conditions, the overall objective being to gain insight into the integrated workings of the albumen in the egg's defence. The search for inhibitors of microbial growth that may also be effective as preservatives of perishable foodstuffs still remains an area of active research. Thus, the possible use of the albumen's defence mechanisms in food preservation was also considered. Indeed this consideration is reflected in the selection of the micro-organisms used in this study.

LITERATURE REVIEW

BIOLOGICAL PERSPECTIVE

When considering the egg and its environment, Needham (1950) discussed the interactions between the embryo and the environment, for example the absorption of water by the egg or the temperature of the egg is influenced by its surroundings. The egg's dependence on the external environment decreased as the egg evolved, thus the eggs of fish, amphibia and the early reptiles are very dependent on the environment whereas those of the later reptiles and birds are almost independent.

The eggs of fish, amphibia and early reptiles are relatively small, consisting of a large mass of yolk surrounded by several thin layers of jelly. The outermost layer is dense, tough and resistant to abrasion (Salthe, 1963) ; the inner layers are more watery and jelly-like. The outermost of these layers may have been the forerunner of the shell membranes, and the inner layers of albumen (Needham, 1931). This soft, flexible integument allows a close contact between the egg and its surroundings especially water or a wet surface. This facilitates the uptake of water and essential ions such as K^+ and Na^+. Indeed, because these eggs do not contain sufficient water at the time of oviposition, its absorption is essential for successful completion of embryogenesis (Needham, 1931).

The eggs of higher reptiles such as lizards and snakes are only slightly modified from those described above. A large mass of yolk is enclosed by a thin layer of albumen (Clark, 1946), external to which is a multi-layered shell membrane (Packard *et al.*, 1977 ; Sexton *et al.*, 1979). In eggs of some species a thin crust of calcareous material is present on the surface of the membranes -

perhaps the forerunner of a calcareous shell? These eggs lack a highly structured shell layer and the membranes can easily be stretched without damage. This is an essential factor if the eggs are to absorb large quantities of water (Packard et al., 1977).

Eggs of reptiles such as crocodiles and some turtles (e.g. trionychids, testudinids) together with those of birds, have a large mass of yolk surrounded by a thick layer of albumen, external to which is a pair of shell membranes and a rigid calcareous shell. The shell arises from nucleation sites on the outer membrane (Schmidt, 1957 ; 1962 ; Packard and Packard, 1979), and is perforated to allow the diffusion of gases and water vapour between the embryo and its environment (Paganelli et al., 1978 ; Packard and Packard, 1979). These eggs are larger than those discussed previously and absorb little (if any) water, either liquid or vapour, during incubation. Indeed, such eggs contain all the water and nutrients for embryo development. When absorption of water is no longer necessary, the need to conserve water assumes importance. This is achieved by the selection of a shell having a water vapour conductance proportional to the minimum requirement of the embryo to obtain O_2 and rid itself of CO_2. Water vapour conductance is determined by the number of pores per shell, shell thickness, length of incubation, temperature, and the steepness of the diffusion gradient existing in the nest. Some water has to be lost from the egg to create an air space of sufficient volume to support pulmonary respiration of the embryo and to provide space for its movement during the hatching process (Rahn et al., 1976). Drent (1975) calculated that on average, eggs in nests lose about 16% of the water present at oviposition.

Ar and Rahn (1978) showed that the incubation period in days (I), egg mass in grams (M) and shell conductance, G (H_2O) are

interrelated :

$$\frac{I.G\ (H_2O)}{M} = 5.13 \pm 0.86 \text{ mg/g (torrs)}$$

if shell conductance and incubation period are known, then the diffusion gradient required to achieve the 16% loss deduced by Drent (1975) can be calculated. Continuous monitoring of the relative humidity of nests (Howey et al., 1977) has shown that the steepness of the diffusion gradient fluctuates markedly during the day but that the average value agrees with that obtained by Rahn et al., (1977) who measured water uptake in the nest by using an egg hydrometer (an empty shell of the species under study filled with dry silica gel). The critical role of egg shell porosity has been questioned by Simkiss (1980) who hatched chicks successfully even though water loss had been accentuated by drilling holes in the broad pole of the shell. Although the chicks were smaller, they had a normal water content and he concluded that the growth of embryos is modulated according to the metabolites available. The shell's mechanism of water conservation and the metabolic control mechanisms of the embryo are both likely to be involved in the successful development of the embryo.

The intense predation of soft-shelled eggs by soil invertebrates and micro-organisms is considered by some (Needham, 1931 ; Packard and Packard, 1980) to be the most plausible reason for the selection of a rigid shell which became progressively thicker and more complex. The shell makes an important contribution to the antimicrobial defence of the egg and is thus an essential component in the breeding success of all birds.

Presumably, there is a requirement for the embryos in the eggs of all oviparous animals to develop in a germ-free environment. Before

an egg shell evolved, the antimicrobial defence probably depended on the albumen or its precursors. The eggs of the salmon (Oncorhynchus) have a "capsule" which is colonized by a characteristic bacterial flora consisting of predominently Cytophaga spp. and the well-being of the embryo is thought to depend on a dynamic ecosystem precluding colonization of the egg by potential pathogens (Bell et al., 1971). The albumen of fish eggs contains substances, "protectins", which are considered to function in the embryo's protection (Uhlenbruck et al., 1972). The jelly surrounding amphibian eggs (Gabayeva, 1962) possesses antimicrobial properties as does the albumen of steppe turtle (Testudo horsfieldii) eggs (Movchan, 1964 ; Movchan and Gabaeva, 1967). Although these reports indicate the antimicrobial nature of the albumen and its precursors, the breeding success of most fishes, amphibia and reptiles is still dependent primarily upon the production of large numbers of eggs to counteract the high rate of macropredation. In contrast, in the evolution of the cleidoic egg, such as that of birds, breeding success is dependent upon a high hatchability of the few eggs produced per pair per breeding season. Presumably, the selective pressures that have favoured the evolution of a few large eggs have also favoured a defence system whereby the large allocation of energy is conserved, and the developing embryo is protected from predation by micro-organisms.

When discussing this topic Tokin (1959) implied that immunological properties of the egg, particularly the albumen, provides a passive immunity. The antibody content of the egg however is confined to the yolk and there is little or no antibody in the albumen. Any antibody in the yolk is restricted to those raised in response to antigens to which the hen has been exposed (Yamamoto and Bigland, 1966) thus, until the immunological defence is formed after hatching, the embryo must rely on another system. Board (1969) suggested that "before the onset

of incubation, the yolk in a cleidoic egg such as that of a bird is shielded from microbial colonization through the combined workings of physical (shell and shell membranes) and chemical (albumen) defence systems". It was noted subsequently that the central location of the yolk and thus the biological structure of the egg also contributes to its defence (Board and Hornsey, 1978). This represents a non-specific system (i.e. one that does not recognize a specific antigen), which like that of mammalian milk, operates in the absence of control systems and yet functions with an efficiency comparable to that of an immunological form.

HISTORICAL PERSPECTIVE

The induction of rotting, by simply shaking eggs, had been used as evidence of spontaneous generation by its supporters. Disagreement with this led Gayon (1873), an associate of Pasteur, to make the first systematic study of the bacteriology of eggs. Gayon could not induce rotting by shaking eggs and he noted that bacteria were always present in rotten eggs. In the following thirty years there were several studies of the microbiology of eggs. Although a few workers observed a high incidence of infected contents (Stiles and Bates, 1912 ; Bushnell and Maurer, 1914) ; the majority agreed that the greater proportion of eggs were sterile (Hadley and Caldwell, 1916 ; Haines, 1938). Haines (1939) attributed such divergent opinions to the technical difficulties of aseptically sampling the viscous albumen. Indeed the failure to resolve this problem led Brooks and Taylor (1955) to make the very general statement that "roughly 90% of newly laid eggs are free from micro-organisms and the true value may be even higher".

The possibility that the egg may be contaminated in the oviduct was discussed in the review by Haines (1939). There is no direct evidence that this occurs. It has been surmised that the oviduct of a healthy hen may become contaminated with micro-organisms (Rettger, 1913 ; Harry, 1963) by (1) the upwards movement of the rectum, or their introduction during insemination (2) from the bloodstream supplying the oviduct. The support for the first view comes from the observation that the oviduct does occasionally undergo violent anti-peristaltic contractions causing feathers and small stones to be included in the albumen of otherwise normal eggs (Romanoff and Romanoff, 1949). As the incidence of such inclusions in eggs is low it can be assumed that this mode of contamination is of academic interest only. Likewise the claim that copulation results in infection of the upper oviduct has yet

to be proved conclusively. In the second case there is evidence that pathogens such as Salmonella spp. pass from the alimentary canal via the blood to the ovaries (Rettger, 1913 ; Gordon and Tucker, 1965). It would seem that general contaminants of the ovaries are present in low numbers only because enrichment methods were required for their isolation (Harry, 1963). Apart from Salmonella spp. other micro-organisms e.g. micrococci recovered from the oviduct have not been associated with rotting of eggs nor the death of embryos in incubating eggs.

The main contamination of the egg occurs after laying as a result of the shell's contact with nest surfaces, harbouring dust, soil and faecal material (Haines, 1939 ; Zagaevsky and Lutikova, 1944 ; Board et al., 1964 ; Moats, 1980). When the composition of the flora of the shell is compared with that of rotten eggs, it is notable that Gram-positive organisms dominate the former and Gram-negative ones the latter (Haines, 1938 ; Board, 1965 ; Board and Board, 1968). The commonest contaminants of the shell are micrococci and other Gram-positive bacteria which are resistant to desiccation (Table 1). The commonest contaminants of rotten eggs are listed in Table 2. It is noteworthy that Gram-negative bacteria are the organisms most frequently recovered from incubating eggs (Pathak et al., 1960 ; Seviour et al., 1972). This suggests that Gram-negative bacteria are better equipped than Gram-positive ones to overcome the antimicrobial defences of the egg.

TABLE 1

TYPES OF MICROORGANISMS PRESENT ON THE SHELL OF THE HEN'S EGG

Type of Organism	Frequency of Occurrence
Streptococcus	1
Staphylococcus	2
Micrococcus	3
Sarcina	1
Arthrobacter	2
Bacillus	2
Pseudomonas	2
'Achromobacter'	2
Alcaligenes	2
Flavobacterium	2
Cytophaga	2
Escherichia	2
Aerobacter	2
Aeromonas	1
Proteus	1
Serratia	1

1 - occasionally present ; 2 - on most eggs but in small numbers ;
3 - always present in large numbers.

(from Board 1968)

TABLE 2

DIFFERENT TYPES OF BACTERIA FOUND IN ROTTEN EGGS

Type of Organism	Frequency of Occurrence
Pseudomonas aeruginosa	1
Pseudomonas fluorescens	3
Pseudomonas putida	3
Pseudomonas maltophilia	2
Flavobacterium	1
Alcaligenes	3
Achromobacter	1
Cytophaga	1
Aeromonas	2
Proteus	3
Escherichia	3
Hafnia	2
Citrobacter	2
Bacillus	1
Micrococcus	1
Streptococcus	1
Arthrobacter	1

1 - present on rare occasions only ; 2 - infrequently ;
3 - commonly.

(from Board 1968)

THE ANTI-MICROBIAL DEFENCE SYSTEM OF THE AVIAN EGG

The contributions made by the major components of the egg to the maintenance of the embryo are listed in Table 3. The vulnerable food store of the egg appears to be protected from infection by invading micro-organisms by a physical and chemical defence, and by the biological structure of the egg.

Physical defence

(i) The shell

The contention (Haines, 1939) that the shell impedes microbial infection of the contents of the hen's egg was based mainly on indirect evidence. The observations pertaining to this were : (a) less than 1% of nest-clean eggs rot during storage (Brooks and Taylor, 1955) ; (b) the levels and incidence of contamination of eggs having cracked shells are greater than those having undamaged shells (Miller and Crawford, 1953) ; (c) the rate and incidence of rotting can be increased merely by cracking eggs before exposing them to rot-producing bacteria (Brown et al., 1966).

The shell of the hen's egg is mainly calcium carbonate (98% by weight) in calcite form, together with traces of magnesium, phosphate, an organic matrix and water (Romanoff and Romanoff, 1949). The inner surface (cone layer) of the shell consists of a large number of conical projections (Plate 1) their apices merging with the outer shell membranes (Tyler and Fowler, 1978). The outer compact layer of the shell (Plate 1), the column or palisade layer, may be covered either completely or partly by a thin layer (Plate 1) of small glycoprotein spheres, the cuticle (Tyler, 1969).

The normal shell of the domestic hen contains 7,000 - 17,000

pores (Tyler, 1953), the diameters of which range from 9 - 35 μ (Romanoff and Romanoff, 1949 ; Tyler, 1956), a size that would not be expected to hinder the movement of bacterial cells. The pores which are aligned radially to the surface of the shell (Plate 1), permit the diffusion of respiratory gases and water vapour (Ar and Rahn, 1978).

Studies of the factors contributing to flooding of pore canals (Board and Halls, 1973ab) and the fine structure of pores (Board, 1974 ; 1975 ; Tullet et al., 1975) led to the concept that avian eggshells are water resistant or water repellent. These properties provide the shell with a barrier against the movement of bacteria. Board and Halls (1973ab) noted that many pores were flooded in the shells of hen, guinea fowl and duck eggs that were cuticle-less at oviposition or that had been rendered cuticle-less by chemical or physical means. There are many reports of increased levels of infection leading to rotting of eggs that were scraped or rubbed with abrasives before challenge with rot-producing bacteria (Haines, 1938 ; Haines and Moran, 1940). Even with an egg having an undamaged cuticle there are upward of 10 - 20 pores (Orel, 1959) which for some unknown reason provide portals of entry for bacteria (Paton and Ayres, 1964 ; Board and Board, 1967). These pores are often referred to as "patent pores".

The available evidence (Haines and Moran, 1940) indicates that micro-organisms remain at the surface of the shell unless some agent promotes their translocation along the pores. Three such agencies have been recognized : (a) infiltration of the pores by fungal hyphae ; (b) flooding of the pores with water drawn in by capillary action and (c) the sucking in of water when a warm egg contracts on cooling. It can be assumed that any agent that causes microbial penetration of the shell results in a heterogeneous population being introduced on

or near the shell membranes.

(ii) The shell membranes

The shell membranes consist of ; (a) an outer membrane which is attached to the shell at the cone layer ; (b) an inner membrane which is attached to the inside of the outer membrane except at the air cell and (c) a limiting membrane which separates the inner membrane from the albumen. The outer membrane has a thickness of about 50 - 55 μ and the inner membrane 15 - 17 μ (Tyler, 1961 ; Lifshitz and Baker, 1964). Electron microscopy has shown that both the inner and outer shell membranes consist of a network of fibres (Plate 2) which lie parallel to the surface of the eggs and which are randomly orientated (Bellairs and Boyde, 1969). The diameter of the outer membrane fibres vary from 0.4 - 3.6 μ whereas that of the inner membrane fibres seldom exceed 2 μ (Bellairs and Boyde, 1969). Each fibre consists of a protein core separated from a glycoprotein mantle (Masshoff and Stolpman, 1961) by a small space (Plate 3) which may contain moderately electron-dense material (Plate 4). The limiting membrane is a layer of homogenously dense material in which the innermost fibres of the inner shell membrane appear to terminate (Plate 5).

The chemical composition of the shell membrane fibres is not fully established. Early studies classified them as keratin due to a high cysteine content (Baker and Balch, 1962). More recently the presence of the cross-linking amino acids desmosine and isodesmosine in the protein core of the fibres (Starcher and King, 1980) has suggested that they are elastin-like (Harris et al., 1980) however isolation of an elastin-like component has proved impossible (Leach, 1978 ; Leach et al., 1981) and it is probable that the protein present in the membranes is unique. The cortex of each fibre has been identified

as mucopolysaccharide (Robinson and King, 1968) containing galactosamine, glucosamine, sialic acid, glucose, mannose and fucose (Cooke and Balch, 1970).

The concept that the shell membranes act as bacterial filters arose from the investigations of Haines and Moran (1940). They replaced the egg contents with a suspension of bacteria and then applied suction to the shell. Fluid drawn through the shell and its membranes did not contain bacteria, but it did so when the shell membranes were removed. This was confirmed by Walden et al., (1956) and Garibaldi and Stokes, (1958). When these workers left the bacterial suspension for 18 - 24 h and then applied suction they found that the fluid was contaminated. These techniques are open to criticism because bacteria were made to traverse the shell and membranes in an opposite direction to which they would normally travel when infecting the egg's contents. The process that results in penetration of the membranes is still unknown but it does not appear to be due to digestion of the membranes by bacterial proteases (Garibaldi and Stokes, 1958 ; Board, 1965).

In an attempt to clarify the contribution of the shell membranes, other workers have used different methods to investigate the possibility that the membranes were acting as filters. These methods included holding the shell with or without one or both of its membranes in a suspension of bacteria and recovering micro-organisms from within the egg (Bean and McLaury, 1959 ; Williams and Whittemore, 1967) and inoculating the air cell and recovering micro-organisms from the albumen (Miller and Crawford, 1953 ; Brooks, 1960 ; Board and Ayres, 1965). In summary, it appears that although the shell plus membranes offers greater resistance to penetration than does the shell alone there is little evidence to support the assumption that the outer structures of the egg are well adapted to resist bacterial penetration.

Chemical defence

(i) The shell membranes

It has been claimed (Brooks and Taylor, 1955) that the membranes contain antimicrobial substances that suppress the growth of micro-organisms. Korotkova (1957) has been quoted in the literature (Board, 1966) as having detected lysozyme in the shell membranes. Owing to difficulties arising over translation from the Russian, she has been misquoted ; the lysozyme she was referring to was that of the underlying albumen.

Stuart and McNally's (1943) observations that there was a rapid death of Gram-negative bacteria in a saline suspension of shell membranes supported the contention that the membrane contained antimicrobial substances. Subsequent studies have shown that micro-organisms isolated from rotten eggs grow when added to shell membranes suspended in a solution of mineral salts, providing the latter were not bactericidal (Board, 1965). The micro-organisms used in this study however were presumably resistant to the antimicrobial defences of the egg anyway to be successful rot-inducing organisms and so the situation remains unresolved.

The colonization of the shell membranes by mixed populations of bacteria has received little study (Seviour and Board, 1972). The limited results indicate that the early phase of infection of the membranes is characterized by a selection of micro-organisms. The Gram-negative members of the flora increase at the expense of the Gram-positive ones. The actual rate of change in these populations is determined by temperature which also influences the composition of the Gram-negative fraction. For example coliforms do not develop in eggs held at room temperature but do so at 37°C (Board and Ayres, 1965).

Many investigators have noted a lag of 15 - 20 days between

penetration of the shells of newly laid eggs and the appearances of large numbers of organisms or macroscopic changes in the albumen (Zagaevsky and Lutikova, 1944 ; Miller and Crawford, 1953 ; Stokes et al., 1956 ; Garibaldi and Bayne, 1962 ; Board and Ayres, 1965). The earlier literature attributes this to the membranes providing a mechanical barrier against microbial invasion ; later investigations indicated that the shell membranes play only a minor role in impeding invasion and that it was the antimicrobial nature of the albumen that was responsible for this lag. Brooks (1960) suggested that growth was restricted because of an iron deficiency and this view was supported by the demonstration (Board, 1964) that considerable bacterial multiplication occurred immediately following the seeding of the air-cell with bacteria suspended in a weak solution of ferrous sulphate.

(ii) The albumen

Albumen, a viscous, heterogeneous material, constituting about 60% of the egg, consists of three layers in freshly laid eggs. Each layer contains different amounts of dissolved material. The approximate percentage of total egg white protein : dry matter for the hen's egg is 25 : 21% in the outer thin white, 57 : 12% in the albuminous sac and 17 : 13% in the inner thin white (Brooks and Taylor, 1955). The albumen differs from most other body fluids in that it consists mainly of proteins (10%) and water (80-90%). The protein content of thin and thick egg white is similar but the latter contains about four times as much ovomucin. The negligible (0.02 - 0.03%) amount of lipid has not been characterized. The free carbohydrate, mainly glucose, amounts to 0.5% of the albumen. There is also a wide variety of inorganic elements in dissociated and bound form. These occur in minute amounts ; their concentration may be influenced by the hen's diet, environment, temperature and age of bird (Powrie, 1977).

The early investigations concerning the microbiology of eggs indicated that there was a higher incidence of infection of the yolk than of the white (Maurer, 1911 ; Stiles and Bates, 1912). In parallel with the studies on the extent of microbial contamination of the egg, there were those that revealed that heavy contamination of the egg did not occur even when the egg was submerged in broth cultures or the white inoculated (Haines, 1939). Wurtz (1890) was probably the first to suggest that egg white was germicidal. He noted that the typhoid bacillus and pyogenic cocci did not survive in albumen. This property of the egg white was confirmed by Laschtschenko (1909) and Rettger and Sperry (1912). In contrast Parascandole (1893) stated without qualification, that bacteria can grow in egg white. When attempting to summarize these pioneering studies of egg microbiology, Hadley and Caldwell (1916) stated that "all that can be said at the present time is that certain species of bacteria, when placed in albumen, survive but a short time".

Laschtschenko (1909) made the first incisive study on the germicidal property of egg white. He observed lysis of vegetative cells and spores of Bacillus spp. in egg white. As this did not occur when the egg white had been heated to 65 - 70°C for 30 min., he postulated the presence of a thermolabile enzyme. This was confirmed by Fleming (1922), who named the enzyme "lysozyme".

Healey and Peter (1925) observed a rapid change in the pH of the egg white during the week following laying. The pH rose from 7.5 to 9.5 due to the loss of CO_2 from the egg through the pores in the shell ; the rate of change was dependent on temperature and p CO_2 of the atmosphere (Sharp and Whitaker, 1927). Sharp and Whitaker (1927) inoculated tubes of fresh egg white, adjusted to values over the range pH 5 - 10, with common contaminants of rotten eggs and found that most of the organisms were unaffected at pH 6 - 8 but were killed

at pH 9 - 10. They suggested that a change in the pH of the albumen may have been responsible for the observations of Parascandole (1893), who found no toxicity with the albumen taken from eggs which had been coated with paraffin-wax immediately after laying. This treatment impedes the diffusion of CO_2 across the shell and the concomittant shift in pH (Sharp and Powell, 1931).

Friedberger and Hoder (1932) noted that micro-organisms that were not lysed by egg white were flocculated by high dilutions of egg white. They suggested that this property should be included among the antimicrobial properties of egg white.

Schade and Caroline (1944) found that the growth of Staphylococcus aureus, Escherichia coli, Shigella dysenteriae and Saccharomyces cerevisiae was inhibited in nutrient broth supplemented with egg white. It occurred when the mixture was adjusted to pH 7.4 or above but not when it was adjusted to pH 5.8 or less. Of 10 vitamins and 31 elements tested, iron alone overcame the inhibition. The iron-binding substance was isolated from egg white by Alderton et al. (1946) and identified as conalbumin, an albuminous protein originally isolated from egg white by Osborne and Campbell (1900). It is now known as "ovotransferrin" and it belongs to a class of proteins, the transferrins, which are present in various fluids of vertebrate animals (Feeney and Komatsu, 1967 ; Feeney and Allison, 1969). Pure preparations of ovotransferrin have also been used to inhibit microbial growth. It has been established that they must be present in stoichiometric excess of the iron found in a medium by chemical analysis (Fraenkel - Conrat and Feeney, 1950 ; Feeney and Nagy, 1952). Ovotransferrin is considered by some to be the principal component of the antimicrobial defence system (Board and Fuller, 1974 ; Board and Hornsey, 1978).

Brooks (1960) was unable to obtain equivalent rates of multiplication of pseudomonads in albumen supplemented with iron and

pseudomonads suspended in a favourable medium. This led him to suggest that an inadequate supply of simple nitrogenous substances might be important, as noted earlier by Haines (1939). The albumen of fresh eggs of domestic hens contains approximately 0.14 - 0.54 μ moles/ml and 2.3 μ moles/ml after storage for several weeks (Ducay et al., 1960). This has often led to the assumption that contaminants of the albumen would be forced to satisfy their nitrogen requirements at the expense of the egg white proteins. Such a view did not receive support from the work by Board (1966) who found that the course of infection of eggs inoculated with proteolytic and non-proteolytic bacteria was indistinguishable.

Biological structure

The growth that follows the lag, noted previously when bacteria are present on the shell membranes, has been attributed to a spontaneous change in the properties of the shell membranes (Elliot and Brant, 1957 ; Brooks, 1960 ; Hartung and Stadelman, 1963). Sharp and Whitaker (1927) noted that multiplication of organisms which invade the albumen does not occur unless they make contact with the yolk. In eggs held at 10°C or less, slow multiplication of the organisms in the shell membranes occurs throughout incubation and this is associated with a slow but progressive increase in the level of contamination of the albumen (Board and Ayres, 1965 ; Board et al., 1968). Eggs held at 10 - 37°C have a slightly different mode of infection. Here the renewed multiplication of the organisms in the shell membranes is associated with a union of the yolk and membranes (Board and Ayres, 1965 ; Board et al., 1968). During storage at ambient temperatures the amount of outer thin white increases due to breakdown of the albuminous sac. The density of the white increases as it loses water by evaporation and absorption by the yolk. Finally,

the yolk rises to make contact with the membranes and extensive multiplication of the micro-organisms can now take place. The viscosity of the albumen and the gelatinous nature of the albuminous sac are important components of the egg's defence. They impede microbial movement (Gillespie and Scott, 1950) to the yolk and also maintain the yolk in a central location, thus keeping it at the greatest distance from the contaminants, restrained by the membranes (Board, 1964).

TABLE 3

THE CONTRIBUTIONS OF COMPONENTS OF THE EGG TO THE MAINTENANCE AND DEVELOPMENT OF THE EMBRYO

Component.	Physiological function.	Physical protection.	Antimicrobial defence.
The integument (preening oils, cuticle/cover, true shell and shell membranes *)	Exchange of respiratory gases. Conservation of water. Reservoir of Ca^{2+} Depot of carbonate. Insulation. Conductance of sound, waves and photo-stimuli.	Protection from crushing. Prevention of water logging. Camouflage.	Barrier to microbial invasion.
Air cell.	Air reservoir and induction of breathing. Water conservation - a 'cold nose' phenomenon.	Compensation for changes in pressure.	-
Albumen.	Reservoir of water Depot of cations (Na^+, Mg^{2+}, K^+) Exchange of O_2, CO_2, H^+ with young embryo. Source of protein.	Cushioning against damage due to sudden movement. Lag against temperature change. Scaffolding for yolk and embryo.	Viscosity an impediment to bacterial movement. Controls rate and extent of microbial growth. Passive immunity of chick
Yolk.	Principal depot of all major and minor nutrients.	Location of young embryo at least distance from heat of brood patches.	Passive immunity of chick.

* Board et al., (1977).

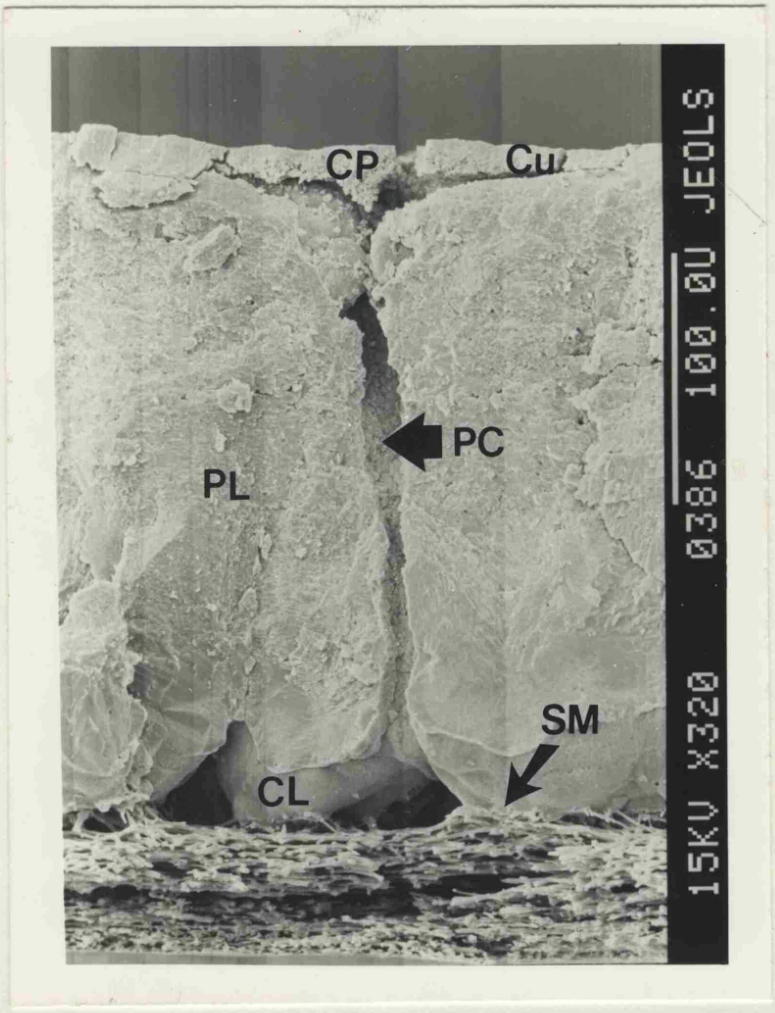
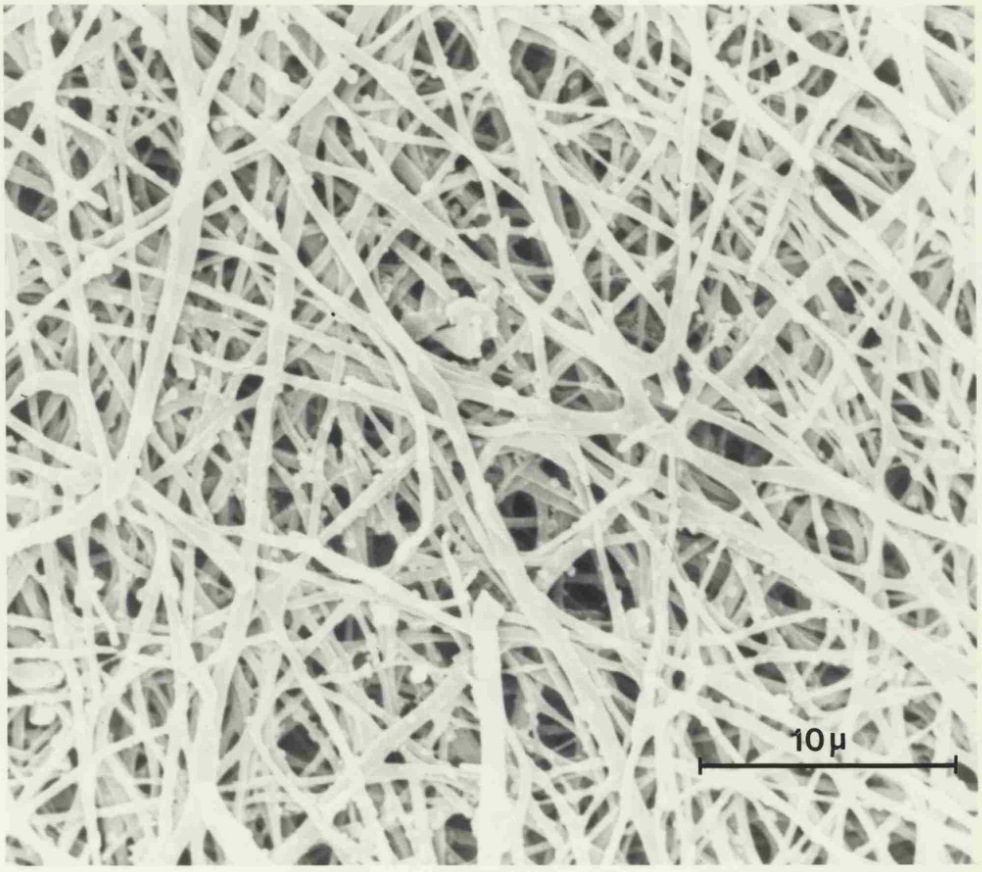


Plate 1. Radially fractured egg shell of the domestic fowl as viewed with the scanning electron microscope showing the cuticle plug (CP), cuticle (Cu), pore canal (PC), palisade layer (PL), cone layer (CL) and shell membranes (SM). The bar marker represents 100 μm.

Plate 2. Scanning electron micrograph of domestic fowl egg shell membranes showing the complicated network of fibres. The bar marker represents 10 μm.

Plate 3. Scanning electron micrograph of domestic fowl egg shell membranes. A broken fibre reveals a central core (C) surrounded by a mantle (M). The space between core and mantle is clearly visible. The bar marker represents 0.5 μm.


10µ

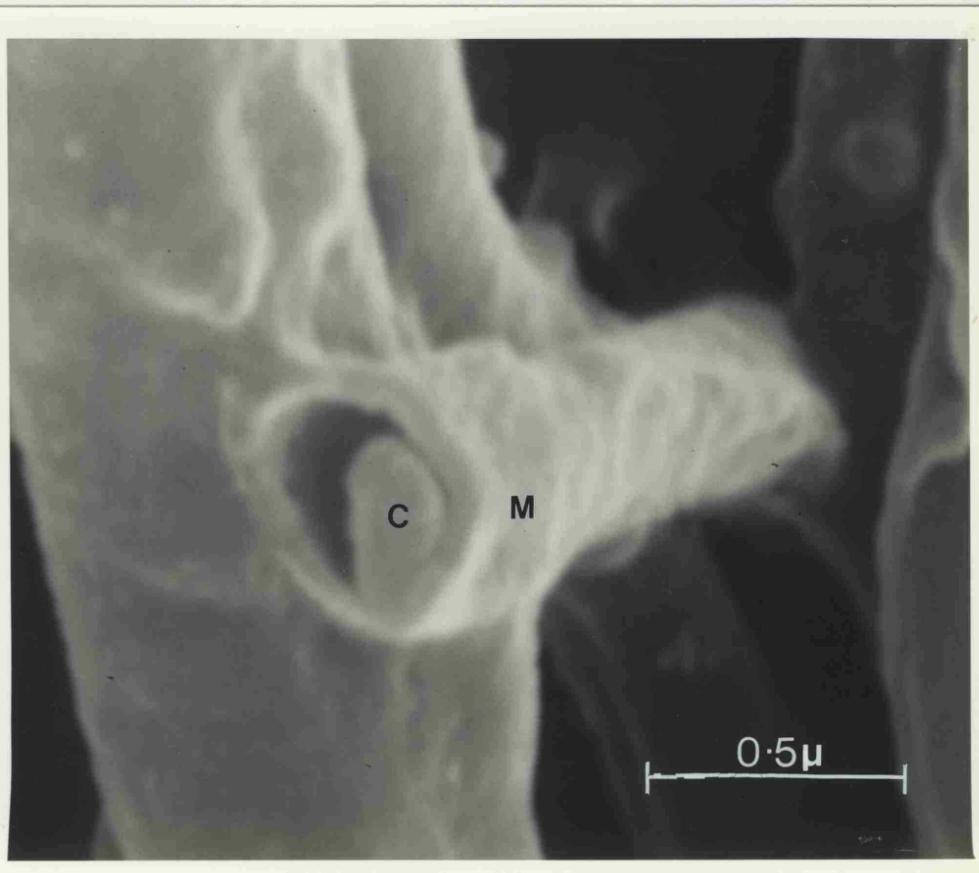
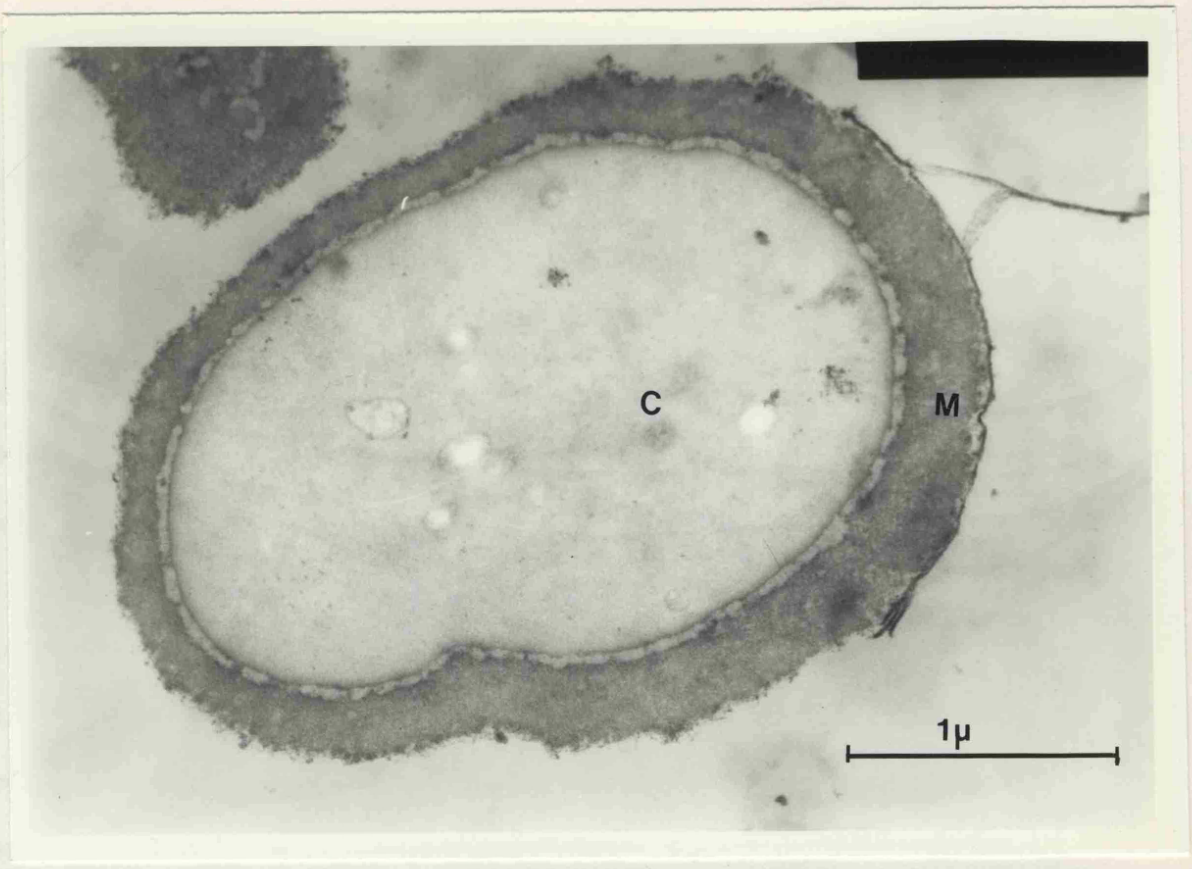
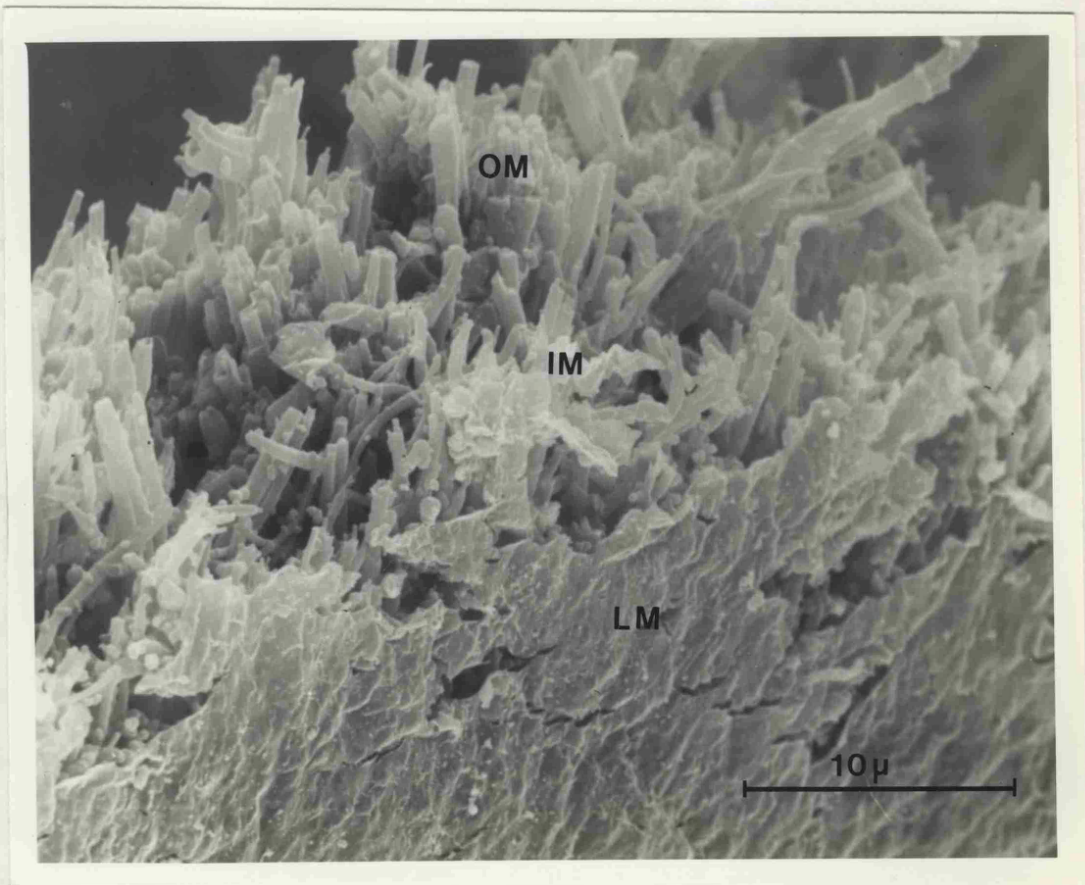

C
M
0·5µ

Plate 4. Electron micrograph of a transverse section through a single fibre in the outer shell membrane of the domestic fowl. The mucopolysaccharide mantle (M) has been stained with 1% (w/v) ruthenium red. The interfibral space is clearly visible between the fibre core (C) and mantle (M). The bar marker represents 1 μm.

Plate 5. Scanning electron micrograph of domestic fowl egg shell membranes showing the three distinct layers of the outer membrane (OM), the inner membrane (IM) and the limiting membrane (LM). The bar marker represents 10 μm.


C
M
1µ


OM
IM
LM
10µ

PHYSICO-CHEMICAL PROPERTIES OF THE PROTEINS INVOLVED IN THE ANTIMICROBIAL DEFENCE SYSTEM OF THE ALBUMEN

The albumen proteins are unique because of their glycoprotein nature and wide range of biological properties. There are about 50 proteins in the albumen which are easily purified because of the lack of interferring materials such as salts, sugars or lipids. Nineteen of these proteins can be detected by starch-gel electrophoresis (Lush, 1961) ; of these, only six exist at a concentration of 1% or higher. Variations in the number of electrophoretic bands of albumen from various species and inbred lines of domesticated birds has been attributed to genetic polymorphism (Lush, 1961 ; Baker and Manwell, 1962). The globulins (Feeney et al., 1963), ovotransferrins (Ogden et al., 1962 ; Ferguson, 1971 ; Frelinger, 1972), ovomucoid (Feeney et al., 1967) and ovoinhibitor (Davis et al., 1969) all possess multiple forms. Sibley (1960 ; 1970) has considered the electrophoretic patterns of avian egg whites to be of value in avian taxonomy.

Many techniques have been used to fractionate and purify egg white proteins (Appendix). Indeed much of the knowledge of the biological nature of albumen has come from the physico-chemical properties of the proteins obtained by these methods. Until recently, these techniques had been applied to the ten major proteins of the albumen (Table 4) of the hen's egg (Osuga and Feeney, 1974) but information relating to their biological properties is perhaps not restricted to the hen because many of these proteins occur widely in birds eggs (Feeney and Allison, 1969).

The physico-chemical properties of those proteins considered to be involved in the antimicrobial defence system of the albumen are

discussed in detail in the following section.

Lysozyme

Although the lysis of bacteria by egg white was first noted by Laschtschenko (1909), it was Fleming (1922) who appreciated fully that lysis was caused by an enzyme, lysozyme, present in most animal and human tissues and secretions, some vegetable tissues, and to a marked extent in chicken egg white. Methods for the purification of lysozyme are given in the Appendix.

Salton (1952) showed that the substrate for lysozyme was the cell wall of Micrococcus lysodeikticus, the organism isolated by Fleming (1922). Confirmation that lysozyme acted on the cell wall of prokaryotes came from the isolation of osmotically fragile protoplasts from Bacillus megaterium (Weibull, 1953) and sphaeroplasts from Escherichia coli (Zinder and Arndt, 1956). Thus identifying the cell wall as the rigid outer layer of the bacterial cell which determines its shape and protects cells from osmotic lysis.

Berger and Weiser (1957) showed that chitin, a linear polymer of β (1-4) linked units of N-acetylglucosamine (NAG), was lyzed by chicken egg white. Subsequently, a tetrassacharide isolated from lysozyme digests of M. lysodeikticus was shown to contain equimolar amounts of NAG and N-acetylmuramic acid (NAM) (Salton and Ghuysen, 1959), β (1-4) linked (Jeanloz et al., 1963 ; Tipper et al., 1965). The cell wall of M. lysodeikticus is formed from a highly water-insoluble polymer, peptidoglycan, which consists of linear strands of alternating units (Fig.1) of NAG and NAM, β (1-4) linked and interconnected by peptide chains (Fig.2) attached to the D-lactyl group of some (50%) of the NAM units. It is the β (1-4) linkages (Fig.1) between the NAM and NAG residues that are attacked by lysozyme (Berger and Weiser, 1957 ; Salton and Ghuysen, 1960). The enzyme is

considered to be a N-acetyl hexosaminidase (E.C. 3.2.1.17) and along with related enzymes is classified as a muramidase. Although no other type of linkage has been demonstrated in peptidoglycans, a micro-organism with a different linkage would be resistant to lysozyme. The absence of N-acetyl groups on the glucosamine in the peptidoglycan of Bacillus cereus (Araki et al., 1972) provides another example of lysozyme resistance - lysozyme sensitivity can be restored to some degree by N-substitution with acetyl, propionyl, butyryl or formyl groups (Amano et al., 1980). With other Gram-positive bacteria resistance to lysozyme may be due to cell wall accessory materials masking the peptidoglycan e.g. Teichoic acid, a highly negatively charged polymer present in the cell walls of Staph. aureus and many other bacteria, can bind a basic protein such as lysozyme and thus confer resistance.

In contrast with Gram-positive bacteria peptidoglycan makes a relatively small contribution (5-10%) to the cell walls of Gram-negative bacteria (Rogers and Perkins, 1968) ; it is buried beneath laminae of lipoproteins and lipopolysaccharides (Fig.3) which provide a barrier to the diffusion of lysozyme. This may be disrupted experimentally by incubation under alkaline conditions (Zinder and Arndt, 1956 ; Vos, 1964), freezing and thawing (Kohn, 1960) or treatment with alkaline (pH8-9) ethylenediamenetetraacetic acid (E.D.T.A.) (Birdsell and Cota-Robles, 1967). There is no evidence that this occurs in albumen (Board and Halls, 1973c) and it is noteworthy that Gram-negative bacteria are the dominant contaminants of rotten eggs (Board, 1964 ; 1965).

Lysozyme (Fig.4), a basic protein with a molecular weight of about 14,000 consists of a single polypeptide chain of 129 amino acids (Jollès et al., 1963 ; Canfield, 1963) cross-linked in four places by disulphide bridges (Brown, 1964). The three dimensional

structure of the molecule was determined by X-ray analysis (Johnson & Phillips, 1965 ; Blake et al., 1965 ; Phillips, 1966 ; 1967). The sequences of amino acid residues 5 - 15 ; 24 - 34 ; 88 - 96 form three lengths of α helix however these appear to be distorted from the "classical" form. The helical sections tend to be rotated so that their - CO - groups point outwards and their - NH - groups inwards from the axis of the helix. This rotation is necessary for the internal hydrogen bonding of the molecule. There are two lengths of anti-parallel pleated sheets which occur between residues 41 - 45 and 50 - 54. Residues 46 - 49 are folded into a hair-pin bend between the two lengths described above. The whole structure is stabilized by hydrogen bonds but the remainder of the chain, other than the residues described is folded in irregular ways that defy simple description.

Phillips (1966) commented on the folding of the lysozyme molecule. The folded chain forms a structure with two wings lying at an angle to each other (Fig.4). The length of α helix formed by residues 86 - 96 lies in a gap formed between the two wings but does not completely fill it. The remaining cleft forms the active site.

Johnson and Phillips (1965) showed that lysozyme was inhibited by the chitin trisaccharide, $(NAG)_3$. It was bound to the enzyme molecule by six hydrogen bonds and over 40 Van der Waal's contacts, filling nearly half the length of the cleft. Inhibitors longer than three sugar residues could not be used because they were more readily cleaved and had difficulty diffusing into the enzyme crystal. Phillips (1966), on the assumption that the interactions involved in the binding of the inhibitor were the same as those involved in the binding of the substrate, constructed a model showing how a substrate consisting of six residues (A - F) of NAG could bind to the cleft. This model showed that : (a) six sugar residues fill the entire length of the cleft ; (b) residue D is distorted from its normal chair conformation

to a half-chair conformation ; (c) N-acetylmuramic acid could not bind to the enzyme at subsites A, C or E due to the D-lactyl ether group at C3 ; (d) the only possible linkage affected by the enzyme was between sugar residues D and E ; (e) the most reactive groups near subsites D and E are the carboxyl groups of GLU 35 and ASP 52 which are located on either side of the β (1-4) linkage. Johnson and Phillips (1965) observed a movement of the side chain of TRP 60, about 0.75Å towards sugar residue B, when the substrate was bound to the enzyme thus indicating an induced fit (Koshland, 1958).

Rupley (1967) showed by ^{18}O-labelling experiments that lysozyme - catalysed reactions involved cleavage between C1 of one pyranose ring and the oxygen joining it to C4 of the next ring. Glycosides are especially susceptible to acid - catalysed carbonium ion formation because the neighbouring ring oxygen stabilizes the positive charge. In glycopyranosides however, a conformational change is necessary for this to occur. The enzymic mechanism has been postulated to take place in the following manner. A lysozyme molecule attaches itself to the bacterial cell wall by interacting with six exposed amino sugars providing other components e.g. teichoic acids do not prevent the enzyme from attaching. During this process, residue D is distorted from its usual conformation. GLU 35 then transfers its terminal hydrogen atom in the form of a hydrogen ion to the glycosidic oxygen bringing about the cleavage of the bond between that oxygen and C1 of residue D. This creates a positively charged carbonium ion (C^+) where the oxygen has been removed from C1. This ion is stabilized by interaction with the negatively charged side chain of ASP 52 until it can combine with a hydroxyl ion (OH^-) that diffuses into position from the surrounding water. The lysozyme molecule then falls away leaving behind a punctured cell wall and a cell that may lyse in hypotonic media. The reactive groups near the bond cleaved are in environments

that favour this catalytic action i.e. ASP 52 is in a highly polar environment and is probably ionic under most conditions - GLU 35 is in a non-polar environment and is unlikely to be ionised. Table 5 lists a few of the many interactions involved in the binding of the substrate to the enzyme.

Sharon and Seifter (1964) and Kravchenko (1967) reported a "transglycosylation" action for lysozyme. This proceeds in the same way as hydrolysis but, instead of water completing the reaction, an acceptor saccharide attaches to the cleft at subsites E and F and is bound to the residues left behind in the cleft.

The decrease in turbidity with time of a suspension of lyophilized cells of M. lysodeikticus is the commonest method for determining lysozyme activity (Parry et al., 1965). Lysis occurs over the pH range of 4 - 10 (Chang and Carr, 1971 ; Davies et al., 1969 ; Saint - Blancard et al., 1970). The rate of lysis is markedly dependent upon ionic strength ; lysozyme is activated by a low and inhibited by high salt concentrations. This inhibition is correlated closely with the cation concentration and charge (Chang and Carr, 1971) so that polyvalent cations are stronger inhibitors than monovalent ones. Davies et al., (1969) suggested that inhibition was due to the high ionic strength disrupting the electrostatic forces that attach the lysozyme molecule to the bacterial cell wall.

Lysozymes have been isolated from a variety of sources (Feeney and Allison, 1969) ranging from bacteria and phages to avian eggs. Their contribution to the total egg white ranges from 3 - 4% (wet weight) in the chicken, other galliformes and anseriformes (Feeney and Allison, 1969) to only trace amounts in penguin eggs (Manwell and Baker, 1973). The amino acid sequences have been determined for several avian lysozymes (Jollès et al., 1976) and partial sequences are known for many others (Jollès et al., 1977).

Initial studies of the lysozyme of White Embden Goose egg white led to the interesting observation that it differed significantly from that of chicken egg white and other homologous avian lysozymes in amino acid sequence (Canfield and McMurry, 1967), enzymic activity (Jollès et al., 1968 ; Arnheim et al., 1973 ; Geoffroy and Bailey, 1975), crystallography (Bott and Sarma, 1975) and immunological reactivity (Arnheim and Steller, 1970 ; Jollès et al., 1976). The goose lysozyme, lysozyme$_g$, has approximately 180 amino acids, 4 half cysteine residues, 2 disulphide bridges and a molecular weight of 19,500 - 21,500. It is a muramidase like the lysozyme from chicken, lysozyme$_c$ (Arnheim et al., 1973) but it is specific for NAM residues whose lactyl group is peptide linked (Dianoux and Jollès, 1967). In contrast to lysozyme$_c$, it has a marked inability to hydrolyse large polymers of NAG (Charlemagne and Jollès, 1967) and is not affected by NAG inhibitors (Jollès et al., 1968).

The lysozyme molecule contains amino acid residues arranged in "sites" to which antibodies may bind (Wilson and Prager, 1974 ; Arnon et al., 1974 ; Ibrahimi et al., 1979). Antisera produced in response to chicken lysozyme will not cross react with goose lysozyme and vice versa. Thus serological methods can be used to demonstrate the presence of these two lysozymes in other egg whites, for example both lysozyme$_c$ and lysozyme$_g$ co-exist (Morgan and Arnheim, 1974) in the egg white of the Black Swan. Available evidence suggests that these are the products of different genetic loci. The egg white of other birds contains only lysozyme$_c$ (Chicken, Peking Duck) or lysozyme$_g$ (Embden Goose). The absence of either enzyme is not a reflection on whether or not the structural genes for these lysozymes are present in the genome of the species ; both genes are present and for some reason only one is expressed in some instances. A tissue survey of the duck revealed both types of enzyme (Arnheim, 1974). The domestic hen

produces both lysozyme$_c$ and lysozyme$_g$ also the former occurring in the egg white and both in the polymorphonuclear leukocytes (Hindenburg et al., 1974)

Although the lytic action of lysozyme in albumen has been demonstrated with lysozyme-sensitive bacteria (Fleming, 1922 ; Garibaldi, 1960) there is no direct evidence that it plays an important role in protecting avian eggs against infection (Board and Fuller, 1974). The Russian workers Korotkova (1957), Tokin (1964) and Movchan (1964) concluded also that this enzyme has but a minor role in the egg's antimicrobial defence. Lysozyme may be involved more directly in the physical structure of the white and hence the physical defence of the egg. The gelatinous structure of the thick white is formed by an interaction between lysozyme and ovomucin, the structural glycoprotein of egg white (Hawthorne, 1950 ; Dam, 1971 ; Cotterill and Winter, 1955 ; Brooks and Hale, 1959 ; Robinson and Monsey, 1972). Hen egg white ovomucin consists of two components (Robinson and Monsey, 1971 ; 1972 ab ; 1975) : ovomucin (M.W. 210,000) containing about 1% (w/w) sialic acid and β ovomucin (M.W. 720,000) which consists of globular subunits and contains 10% (w/w) sialic acid. Kato et al., 1975 showed that there was an electrostatic attraction between the negative charges of the terminal sialic acid and the positive charges of the lysyl ε-amino groups of lysozyme and that this interaction decreased correspondingly to enzymatic removal of the sialic acid by neuraminidase.

The internal quality of the egg has been related to the lysozyme content of thick egg white (Sauter and Peterson, 1972 ; Goel et al., 1975) thus the contribution of lysozyme to the antimicrobial systems of the egg white can be considered in two ways : (a) lysis of sensitive organisms ; (b) maintenance of the albuminous sac and hence the biological structure of the egg.

Ovotransferrin

The transferrins, a family of homologous proteins, are distributed widely in the sera of most vertebrates (Zsochke and Bezkorovainy, 1974) in the milk and other secretions of mammals (Masson and Heremans, 1971) and in the white of avian eggs (Baker, 1968 ; Sibley, 1970 ; Clark et al., 1963). No particular nomenclature has been generally accepted for these proteins and many names are used in the literature :

Iron binding protein from serum	: transferrin ; siderophilin ; β_1-metal combining protein.
Iron binding protein from milk	: lactotransferrin ; lactoferrin ; red protein.
Iron binding protein from egg white	: conalbumin ; ovotransferrin.

A characteristic feature of all of these proteins is their ability to bind two ferric iron atoms per protein molecule (Aisen et al., 1966) at two different sites (Aasa et al., 1963) to produce a salmon-pink complex (Schade et al., 1949) with an absorption maximum at 400 - 470 nm ; this forms the basis of many assays for these proteins.

The transferrins may form complexes with other transition metals ; these have absorption maxima at different wavelengths and absorbances of different magnitude. Thus, copper yields a yellow complex with transferrin (Holmberg and Laurell, 1947) and ovotransferrin (Fraenkel-Conrat and Feeney, 1950) which has an absorption maximum at 440 nm, but the zinc-ovotransferrin complex is colourless (Warner and Weber, 1953). Displacement studies (Tan and Woodworth, 1969) showed the following relative stabilities of metal-ovotransferrin complexes :

$$Fe^{3+} > Cr^{3+} \quad ; \quad Cu^{2+} > Mn^{2+} \quad ; \quad Co^{2+} \quad ; \quad Cd^{2+} > Zn^{2+} > Ni^{2+}$$

the metal complexes dissociate in acid ($<$ pH6.5) but not in alkaline solution (pH9-10). Divalent iron is not bound to transferrin (Gaber

and Aisen, 1970) although Bates et al., (1973) claim that transferrin possesses ferroxidase activity and that Fe^{2+} is bound to transferrin before being oxidised by atmospheric oxygen.

Starch gel electrophoresis has revealed that ovotransferrin exists in multiple forms. This is particularly notable in the starch-gel patterns of the Ratites (Osuga and Feeney, 1968). The cassowary, for example, possesses six forms of ovotransferrin (Clark et al., 1963) which can be separated one from another. Lush (1961) and Ogden et al. (1962) showed that chicken egg white ovotransferrin varied electrophoretically and genetically within one strain. Methods for the purification of ovotransferrin are given in the Appendix.

Ovotransferrin is a glycoprotein constituting about 13% of the total protein content of hen egg white. Its carbohydrate moiety consists of 3.5 moles mannose and 5.6 moles of glucosamine per 80,000 g of protein (Williams, 1968) ; most (88%) of this carbohydrate occurs as a single oligosaccharide. Ovotransferrin, unlike transferrin, contains no sialic acid (Williams, 1962).

Conflicting observations have been made by workers who have attempted to define the primary structure of ovotransferrin. The amino acid composition is not unusual and does not vary appreciably between ovotransferrins from different avian species (Osuga and Feeney, 1968). Its high molecular weight (88,000 - 95,000) led to suggestions that it is formed from major subunits. The chemical techniques of Elleman and Williams (1970) and the physical ones of Greene and Feeney (1968) and Bezkorovainy et al., (1968) have revealed however that ovotransferrin consists of a single polypeptide chain. Greene and Feeney (1968) suggested that this chain contained two homologous sections each of which originated by gene duplication. This was supported by the observations of Phillips and Azari (1971) who split ovotransferrin into three polypeptide fragments by cyanogen bromide cleavage whereas nine

fragments would have been expected from the methionine content of the protein. The sum of the molecular weight of these fragments (21,000 : 9,400 : 7,000) was exactly half that of the native protein. Other workers have obtained results that suggest ovotransferrin does not consist of identical halves. Thus, Bezkorovainy and Grohlich (1974) split ovotransferrin into eight and Tsao et al., (1974 abc) four, which was increased to eight after reduction followed by carboxymethylation. The latter workers could account for all eight methionine residues and they isolated a fragment, CF1, which had specific iron binding activity. The other binding site was associated with the remaining three fragments, CF2, CF3 and CF4.

Unlike haemoglobin, ovotransferrin does not have prosthetic groups involved in the binding of Fe^{3+}. It was postulated initially that the metal binding site in ovotransferrin is associated with the side groups of specific amino acid residues (Fraenkel-Conrat and Feeney, 1950) and that these were similar for each of the two sites. As the binding of iron and copper to ovotransferrin was shown to be accompanied by the release of three and two protons respectively, Warner and Weber (1953) and Wishnia et al., (1961) suggested that, with the pH at which they worked, tyrosyl groups would be the most likely source of these protons. This was confirmed by the demonstration that no colour developed when iron or copper was added to ovotransferrin which had its tyrosine groups iodinated (Azari and Feeney, 1961) ; there was no loss of colour when the metal-complex was iodinated. Acetylation (Komatsu and Feeney, 1967) and nitration (Line et al., 1967) of ovotransferrin confirmed the contention that tyrosines were involved in the metal-binding site. Indeed with the last two methods, six fewer tyrosyls were modified in the iron-saturated than in the iron-free protein thereby suggesting that three tyrosyls were involved in the binding of each iron atom. Line et al., (1967) and Bezkorovainy and

Grohlich (1971), who used bromoacetate to modify the imidazole groups in transferrin, found that apotransferrin (metal-free transferrin) could not bind iron. Two imidazole groups were found to be bonded to each iron atom.

Schade et al., (1949) were the first to demonstrate that CO_2 was involved in the formation of iron complexes of human serum transferrin. Warner and Weber (1953) showed that ovotransferrin required carbonate or bicarbonate for the formation of the coloured complex. Iron binding to the specific sites on the transferrins needs concomittant anion binding (Bates and Schlabach, 1975) ; carbonate of bicarbonate is the preferred anion but small molecular weight compounds possessing two or more carboxyl groups (oxalate, E.D.T.A.) will also activate the metal binding site (Aisen et al., 1967 ; Woodworth et al., 1975). The anion is necessary for chromophore development (Aisen et al., 1967) and blocking the carboxyl groups with glycinamide prevents or glycine ethyl ester, slows down colour formation (Woodworth et al., 1975). Aisen et al., (1967) who showed that ternary complex formation between metal, chelate and transferrin took place in the absence of bicarbonate, suggested that bicarbonate, through the displacement of the chelate was involved in the rate-limiting step of chromophore development. This view was supported by the kinetic data obtained from the binding of iron and copper to hen ovotransferrin (Phelps and Antonini, 1975). Bates and Schlabach (1975) concluded however that transferrin appears to have no affinity for iron in the absence of anions. The anions serve as binding ligands between the metal and protein, stabilizing the bond and protecting it from hydrolysis (Williams and Woodworth, 1973). Although a model (Fig.5) of the iron binding site involving the anion has been proposed (Schlabach and Bates, 1975), it does not account for other ligands such as the hydroxyls of tyrosine and the imidazole nitrogen of histidine involved in the binding site.

The two iron-binding sites of ovotransferrin were considered to be equivalent and independent of each other (Aisen et al., 1966). Measurement of the binding by equilibrium dialysis (Warner and Weber, 1953) showed that the binding constants for iron are very large (about 10^{30}) and that the two stoichiometric binding constants do not differ appreciably (Aisen and Leibman, 1968). Aisen et al. (1970) proposed the existence of ovotransferrin in three forms at equilibrium : (a) iron free (b) complexed with one Fe^{3+} and (c) complexed with two Fe^{3+}. The existence of these forms were confirmed by Donovan et al. (1976). They found that in the absence of low molecular weight chelators such as nitrilotriacetic acid (N.T.A.) iron binds randomly to the two sites. In the presence of N.T.A. binding at one site is slightly favoured and once iron had been bound at either site the binding affinity for the unoccupied one was decreased. This "anti-cooperativity" may be attributed to a conformational change in the protein following the binding of the first Fe^{3+} (Emery, 1969). Unlike human serum transferrin where marked differences in the release of the first and second Fe^{3+} ions occur (Fletcher and Huehns, 1967 ; Harris and Aisen, 1975), the release of iron by ovotransferrin to chick embryo erythrocytes does not distinguish between the two sites (Williams and Woodworth, 1973).

The iron complexes of ovotransferrin are more stable than the metal-free ones to denaturation by heat, organic solvents, high pressures and exposure to high concentrations of urea or guanidine (Azari and Feeney, 1958 ; 1961). This enhanced stability has been interpreted on the basis that chelation of the metal causes the formation of new bonds that stabilize and perhaps modify the secondary and tertiary structures of the molecule. The formation of the iron complex stabilizes the active centre for iron binding so that the capacity is protected but also that modification of other parts of the molecule can occur without denaturation.

Apart from inhibition of microbial growth, the biological function of ovotransferrin in the avian egg has not been demonstrated. There is no direct evidence for iron-transport by ovotransferrin in egg white ; essentially all the iron of the egg is in the yolk.

Avidin

The ability of certain foods and extracts to protect rats against the harmful effects of raw egg white (Boas, 1924 ; 1927) led Györgi (1939) to the identification of "vitamin H" with the yeast growth factor, biotin (Györgi et al., 1940). The inimical factor of egg white, avidin, isolated by Eakin et al. (1940 ; 1941), formed a non-digestible (Parsons et al., 1940) complex with biotin thus making the vitamin unavailable to the host (Eakin et al., 1940). It is present in the egg white of many birds (Hertz and Sebrell, 1942 ; Jones and Briggs, 1962), the egg jelly of frogs (Hertz and Sebrell, 1942) and the albumen-secreting tissues of the oviduct of laying hens (Fraps et al., 1943). It can be induced by progesterone in the oviduct of oestrogen-primed chicks (Hertz, 1949 ; Fraps et al., 1943 ; O'Malley et al., 1969) but it is absent from the oviduct of non-laying hens and mucosal scrapings of pig, cow and guinea pig oviducts (Hertz, 1946). An avidin-like protein, streptavidin, in the culture filtrate of Streptomyces spp. (Chaiet et al., 1963) also possesses antimicrobial activity which can be reversed by the addition of biotin (Tausig and Wolf, 1964 ; Miller and Tausig, 1964). Biotin-binding proteins have also been found in the egg yolk (White et al., 1976) and plasma (Mandella et al., 1978) of the hen. These are similar to avidin except in their capacity to release biotin. The yolk and plasma proteins are normally saturated with biotin which exchanges quite readily at the body temperature of the hen, an important feature if biotin is to be available for the developing embryo. In contrast, avidin, present in

an unsaturated state in the white, binds biotin very tightly and will not exchange it appreciably at body temperature. This is a significant factor if it is to function as an antimicrobial agent.

Avidin (see Appendix for purification), a basic glycoprotein (M.W. 70,000), consists of four identical polypeptide subunits containing 129 amino acids (DeLange 1970 ; Green and Toms, 1970), with alanine and glutamate at the amino and carboxyl ends respectively. Trypsin and Cyanogen bromide (CNBr) cleavage have established the complete amino acid sequence (DeLange, 1970 ; DeLange and Huang, 1971 ; Huang and DeLange, 1971). The protein is characterized by its high threonine content, single residues of histidine and tyrosine and two of cystein. It also contains 4 - 5 residues of mannose and 3 - 4 of glucosamine linked by one of the acetylglucosamine residues to ASN 17 ; the structure of this carbohydrate is unknown.

Avidin binds four molecules of biotin, one per subunit. Measurement of the dissociation constant by equilibrium dialysis with radioactive biotin (Launer and Fraenkel-Conrat, 1951) gave an upper limit of 10^{-10}M. An accurate determination was impossible because of impurities in the biotin. The rate of exchange of bound ^{14}C-Biotin with an excess of unlabelled biotin by separating free from bound biotin with CM - cellulose gave a value of 10^{-15}M (Green, 1963a).

Fraenkel-Conrat et al. (1952b) concluded that the binding site of avidin did not contain reactive amino, phenolic, imidazole, carboxyl or disulphide groups. They found that significant (>70%) inactivation resulted from oxidation with H_2O_2 in the presence of Fe^{2+}, treatment with formaldehyde in the presence of alanine or NH_2OH at 50°C. Although precise interpretation of these observations was not possible, modification of tryptophan residues was implicated. Oxidation of tryptophan by N-bromosuccinimide indicated that each molecule of biotin protects four tryptophan residues from oxidation, and that each subunit

acts independently to the others (Green, 1963 ab ; Green and Ross, 1968). Tyrosine and tryptophan account for 96% of the absorbance of avidin at 282 nm. The shift of this spectrum to longer wavelengths when avidin reacted with biotin also suggested that tryptophan residues were involved in the binding (Green, 1962). The shift was interpreted as a transfer of tryptophan into a less polar environment on binding to avidin. A lysine residue has also been implicated in the binding of biotin to avidin (Green, 1975).

Biotin analogues also produce changes in the difference spectra on binding to avidin and their dissociation constants can be calculated from the spectrophotometric titration curves (Green, 1963a). The dissociation constant decreases as the similarity of the analogue to biotin becomes more remote. This provides clues about the different parts of the biotin molecule required for binding. For example analogues containing a broken imidazolidone ring have dissociation constants 10^7 times greater than that of biotin. Measurement of the free energy associated with the binding of biotin and its analogues to avidin indicate that every atom in the biotin molecule is involved in the interaction with avidin.

Avidin, and particularly the avidin-biotin complex, have a marked stability to denaturation by heat or breakdown by proteolytic enzymes (Györgi and Rose, 1943). The ionic strength of the heating medium is important ; the release of biotin from avidin by autoclaving (Pai and Lichstein, 1964 ; Wei and Wright, 1964) was more rapid (88% complete after 10 min at 100°C) in the absence of 0.2M Ammonium carbonate than it was in its presence (10% after 15 min at 100°C).

Avidin resist unfolding in high concentrations (8M) of urea (Fraenkel-Conrat et al., 1952a) and moderate concentrations of Guanine hydrochloride (Green, 1963c). Above 3.5M GuHCl, the protein begins to unfold and its binding ability decreases concomittantly. Molecular

weight measurements at 6M GuHCl indicate that the protein dissociates into monomers. Tertiary structure and binding ability are not regained until the concentration of GuHCl is lowered to 2M. Although at 3.5M GuHCl there is no detectable tetramer structure, biotin is bound to refolded monomers. Saturation of avidin with biotin prevents unfolding even in 8M GuHCl or 0.1M HCl. In partially saturated avidin only the unoccupied subunits are vulnerable to denaturation by GuHCl (Green and Toms, 1972) or oxidation by N-Bromosuccinimide (Green and Ross, 1968). The denatured subunits dissociate into monomers and the protected ones recombine to form tetrameric avidin-biotin complexes. This indicates that the binding site is almost certainly situated within a subunit rather than at an interface between subunits. Green and Toms (1973) showed that biotin could be bound to monomers coupled to s Sepharose matrix to prevent them from reassociating. Formation of active subunits was not dependent on interactions with other subunits but interaction was required for firm binding of biotin.

Studies of fluorescence quenching (Green, 1964) have shown that binding to avidin is a random process and that there is no detectable interaction between the sites. Optical rotation and optical rotatory dispersion studies (Green, 1962 ; Green and Melamed, 1966) indicate that binding does not result in any gross morphological changes of avidin. This is supported by the observations (Green and Joynson, 1970) that crystals of avidin and the avidin-biotin complex are isomorphous.

Cross-linking of avidin molecules with bisbiotinyldiamines (Green et al., 1971) has been used to determine the spatial relationships of the binding sites in the subunits. The binding sites are grouped in two pairs at opposite ends with a dimension of 55Å x 55Å x 41Å.

The early methods of estimation of avidin were based on its ability to deprive biotin-requiring micro-organisms such as yeasts (Hertz, 1943)

or Lactobacilli spp. (Wright and Skeggs, 1944 ; Landy et al., 1942) of biotin. Although extremely sensitive these methods were time consuming. As there is an appreciable spectral change (a new absorption band appears ε_{500}= 34,000) when the dye 4-hydroxybenzene -2- carboxylate is bound to avidin (Green, 1965), the amount of avidin may be calculated directly from the absorbance at 500 nm or by using the dye as an indicator in a spectrophotometric displacement from avidin with free or enzyme bound biotin. Use of ^{14}C-biotin is used widely in assay systems. ^{14}C-biotin is added to avidin and any free vitamin separated by absorbing the complex onto CM-cellulose (Green, 1963a), sephadex gel (Wei and Wright, 1964) or precipitating it with antibody (O'Malley and Korenman, 1967). The unabsorbed ^{14}C-biotin can then be determined. A disadvantage of such methods is that other biotin-binding factors, or endogenous biotin present in the tissue samples, will compete with ^{14}C-biotin. This problem can be overcome by methods such as radioimmunoassay of avidin with ^{125}I (Kulomaa et al., 1978).

Ovoflavoprotein

Ovotransferrin was reported to contain riboflavin by Bain and Deutsch (1948). Mahler and Elowe (1954) also suggested that riboflavin was bound to the iron-ovotransferrin complex. Rhodes et al. (1958 ; 1959) noted that riboflavin was the flavin moiety in a flavoprotein isolated from egg white, one mole of riboflavin binds to one mole of apoprotein. As such it differs from most of the naturally occurring flavoproteins which have FMN or FAD as their flavin moiety (Wellner, 1967). FMN and FAD bind weakly and are displaced by riboflavin (Rhodes et al., 1959). Flavoproteins containing riboflavin in the serum of the hen (Winter et al., 1967) and egg yolk (Ostrowski et al., 1962 ; 1968) are seriologically identical (Farrell et al., 1970) to that present in the white.

All of the riboflavin present in the egg white is bound to the apoprotein. The apoprotein is stable to heat (100°C/15 min), but is denatured by proteolytic enzymes and 3M urea. The flavoprotein, unlike free riboflavin, shows little fluorescence at 500 nm.

The apoprotein, a glycoprotein with a molecular weight of 32,000 (Farrell et al., 1969), has a large number of aspartate and glutamate amino acid residues which endows the protein with a strong anionic character as demonstrated by its electrophoretic mobility and absorption onto DEAE-cellulose. The protein is highly cross-linked by eight disulphide bridges ; there are no free sulphydryl groups and all of the sulphur occurs as methionine or cystine (Farrell et al., 1969). The apoprotein contains 14% carbohydrate made up of mannose, galactose and glucosamine (Farrell et al., 1969). Its content of phosphorus (0.8%) can be removed by potato acid phosphatase (Rhodes et al., 1959) without affecting the flavin-binding capacity.

Guanine hydrochloride (5M) causes reversible inactivation of the protein. The $S_{20}W$ changes from 3.05 in 0.02M NaCl to 1.75 in 5M GuHCl suggested fragmentation into separate polypeptide chains or unfolding of a single chain to a less symmetrical molecule (Farrell et al., 1969). Reduction followed by alkylation indicated that the apoprotein consisted of two subunits joined probably by two thiocovalent linkages (Claggett, 1971). The larger unit, which was isolated by Cotner and Claggett (1972), has a molecular weight of 24,000 g/mole and contains five of the dithio linkages.

Chemical modification of specific amino acid residues have been used in attempts to identify the residues involved in the binding site. The use of 1-tosylamido -2- phenylethylchloro-ketone (TPCK) and iodine modification of histidine and tyrosine residues respectively did not affect the binding capacity of the apoprotein. As oxidation caused a decreased absorption at 280 nm and an increase at 330 nm it would appear

that a tryptophan residue is involved in binding. The pH-activity curve (Farrell et al., 1969) closely resembles a titration curve for a carboxyl group with a pK_a of approximately 3.8 thereby suggesting that a carboxylate ion is essential for binding also.

The binding of riboflavin to apoprotein is not influenced by pH ; at pH 4.2, the flavoprotein is dissociated but recombines above this value. Modification of the isoalloxazine ring of riboflavin at the 3 and 9 positions decreases the binding to the apoprotein (Rhodes et al., 1959). This suggests that riboflavin is either bound by several sites or that the entire molecule fits into a specific structure or crevice on the protein.

The content of riboflavin in the egg white of domestic hens reflects that present in the diet (Heiman, 1935 ; Norris and Bauernfiend, 1940). The duck, goose and Adelie penguin do not secrete riboflavin in the egg white regardless of the presence of high levels of the vitamin in the diet (Rhodes et al., 1959 ; Feeney et al., 1968).

Clagett (1971) suggested that the riboflavin-binding proteins of the albumen, yolk and serum were all under the control of a single gene. The blood flavoprotein is transferred directly to the yolk but that in the albumen must arise from de novo synthesis in the oviduct. Although the effect of the altered gene, "rd" is unknown, he proposed that "Rd" may runction through the conversion of a riboflavin binding protein precursor, pro-RBP, to the active compound in a similar way to that in which proinsulin and prochymotrypsin are converted to insulin and chymotrypsin respectively.

The apoprotein of egg white can be assayed directly by titration with a standard solution of riboflavin to the first visible fluorescence with a U.V. light as the exciting source. Fluorescence is quenched completely until a stoichiometric amount of riboflavin is added to the apoprotein. This rapid and inexpensive method gives a sharp end point.

The determination of unbound riboflavin after the addition of riboflavin to apoprotein provides an indirect method of assay. The amount of apoprotein can be calculated from the bound riboflavin. Free riboflavin can be determined spectrophotometrically at 450 nm or microbiologically using a riboflavin-requiring organism such as Lactobacillus casei (Snell and Strong, 1939 ; Silber and Mushett, 1942).

Although the inhibition of the growth of Streptococcus pyogenes and L. casei has been demonstrated in vitro when 10 moles of apoprotein was present for every mole of riboflavin (Rhodes et al., 1959), the half saturation of the apoprotein in the hen's egg white would in theory endow this protein with only a minor role in the eggs antimicrobial defence (Baker, 1968). In practice however micro-organisms in egg white may not be able to obtain sufficient vitamin in the presence of other deficiencies such as iron, avidin, simple molecules of nitrogen and at a high pH.

Proteolytic enzyme inhibitors

Ovomucoid

Ovomucoid was shown by Lineweaver and Murray (1947) to be the proteolytic enzyme inhibitor (Balls and Swenson, 1934 ; Meyer et al., 1936) of chicken egg white. The heterogeneous nature of ovomucoid was demonstrated by attempts to isolate pure material (Appendix). With some of the initial preparations the heterogeneity was due to contamination with ovoinhibitor and lysozyme (Matsuhima, 1958 ; Feeney et al., 1967). Even when these were removed, differences in sialic acid (Rhodes et al., 1960 ; Feeney et al., 1967) carbohydrate content (Feeney et al., 1967) and minor variations in amino acid composition of ovomucoid were demonstrated (Feeney et al., 1967).

Ovomucoid, a glycoprotein (M.W. 28,000), accounts for 11% of the total protein content in chicken egg white. The amino acid composition

of several avian ovomucoids (Osuga and Feeney, 1968 ; Osuga et al., 1974) resemble those of other proteolytic enzyme inhibitors in that there is no tryptophan, little or no methionine but large amounts of cystine. The amount (up to 25% w/w) of carbohydrate in ovomucoid contains : 1 - 1.5% D-galactose ; 4.3 - 4.7% D-mannose ; 12.5 - 15.4% 2-amino - 2 - deoxy - D - glucose ; 0.4 - 4.0% sialic acid and 6 - 9% total hexose. The carbohydrate moiety, which consists of three oligosaccharides, is attached to the protein via a N-(β aspartyl N-acetylglucosaminyl) amino link between glucosamine and asparagine (Monsigny et al., 1968). The carbohydrates role has not been established ; the removal of the sialic acid by neuraminidase does not affect its inhibitory activity (Feeney et al., 1960).

There are three different types of ovomucoid in avian albumen (Rhodes et al., 1960 ; Nakamura et al., 1966) : one inhibits trypsin only or primarily ; another inhibits chymotrypsin only or primarily and a third inhibits both (Fig.6). The last category may be subdivided further ; those that inhibit equimolar amounts of trypsin and chymotrypsin and those that inhibit two moles of trypsin to one mole of chymotrypsin. Although the binding sites for trypsin and chymotrypsin differ (Rhodes et al., 1960) they may or may not overlap such that the binding of one enzyme may influence the binding of the other. Chicken ovomucoid shows a marked specificity ; it inhibits bovine (Feeney et al., 1963) and porcine (Vithayathil et al., 1961) but not human trypsin (Feeney et al., 1969).

Inhibition involves a highly associated enzyme-substrate (inhibitor) complex in which substrate recognition of a specific amino acid is considered to be the initial event. There are two types of trypsin inhibitor, those that lose their inhibitory activity upon modification of (1) their lysine or (2) their arginine residues (Table 6). Thus Stevens and Feeney (1963) showed that acetylation or carbamylation of

the lysine residues of turkey ovomucoid destroyed its inactivation of trypsin but not of chymotrypsin. This procedure had no effect on chicken or golden pheasant ovomucoids activity against trypsin or chymotrypsin respectively. It did however destroy the pheasant's very weak activity towards trypsin. Arginine modification with 1,2 - cyclohexadione (Liu et al., 1968) led to the loss of activity of chicken ovomucoid. Removal of these substrate-like residues by treatment with carboxypeptidase B (Ozawa and Laskowski, 1966) eliminated activity completely. Substrate-like residues necessary for the inhibition of enzymes other than trypsin for example chymotrypsin, have been difficult to identify because the residues involved are frequently either unreactive or modification did not eliminate their substrate-like character.

Dissociation constants for these enzyme-inhibitor complexes are frequently less than 10^{-8} and may be as low as 10^{-13}(Vincent and Lazdunski, 1972). This remarkable stability led several workers to suggest that a covalent bond, formed by acylation of the enzyme's serine by the arginyl carboxyl group of the inhibitors, was the driving force and a possible catalytic intermediate in the inhibition (Laskowski and Sealock, 1971). These suggestions have been questioned by others because direct confirmation of this acyl linkage has not been possible and also because inactive derivatives of trypsin and chymotrypsin form strong specific complexes with several of the ovomucoids (Ryan and Feeney, 1975). These observations indicate that the main strength of interaction is due to many weak non-covalent interactions such as hydrogen bonding and Van der Waals forces (Feeney, 1971). These interactions are stabilized by a close, complementary fit corresponding to a good enzyme-substrate complex. Some covalent bonding may be involved but, contrary to earlier proposals, it is unnecessary for the formation of, and does not add to, the stability of these complexes

(Means et al., 1974).

Ovomucoid shows unusual heat stability and resistance to high concentrations of urea in neutral or acid conditions ; more than 90% of its activity remained after 30 min at 80°C in 9M urea between pH 3-9 but only 6% remained at pH 9 (Stevens and Feeney, 1963). Turkey and pheasant ovomucoids are even more stable to heat.

Ovoinhibitors

Ovoinhibitor, the other main protease inhibitor in hen egg white, was isolated by Matsushima (1958) who found it inhibited bovine trypsin and proteases from B. subtilis and Aspergillus spp. The similarity in structure of chymotrypsin to the bacterial protease subtilisin, promoted studies of the effect of this inhibitor on chymotrypsin (Rhodes et al., 1960). This led to the observation (Feeney et al., 1963) that the weak inhibitory activity against chymotrypsin reported for chicken ovomucoid was due to the presence of ovoinhibitor as a contaminant. Details of the separation of ovoinhibitor from ovomucoid are given in the Appendix.

Like ovomucoid, ovoinhibitor exhibits heterogeneity due primarily to the charge on the molecule which is reflected during purification with starch-gel electrophoresis or DEAE-cellulose chromatography (Tomimatsu et al., 1966 ; Davis et al., 1969). The various forms are similar in their amino acid composition and antiproteolytic activity.

Chicken ovoinhibitor, a glycoprotein with a molecular weight of about 46,500 (Tomimatsu et al., 1966), comprises about 0.1% of the total protein in egg white. Its amino acid composition resembles that of ovomucoid (Davis et al., 1969) and its tryptophan residues are < 1. There are 34 half cystine residues and low amounts of methionine. The content carbohydrate, as yet uncharacterized, is smaller than in ovomucoid.

Chicken ovoinhibitor is considered to be a "double-headed"

inhibitor ; one mole of inhibitor combines simultaneously with two molecules of trypsin and two of chymotrypsin (Tomimatsu et al., 1966). The absence of competition between these two is evidence that separate sites are involved. Competition is evident however when ovoinhibitor is incubated with chymotrypsin and subtilisin suggesting that the two enzymes compete for the same or closely related sites (Tomimatsu et al., 1966). Ovoinhibitor reacts with trypsins and chymotrypsins of bovine (Rhodes et al., 1960) avian (Ryan et al., 1965) but not human origin (Feeney et al., 1969). It differs from most ovomucoids in that arginine is the essential substrate-like residue for binding trypsin (Liu et al., 1971).

Ovoinhibitor shows appreciable stability in acid solution ; 93 - 95% of its activity is retained during 15 min at 90°C, pH 3 - 5, or for 24h at 40°C, pH 2 - 3, or 3h at 40°C, pH 1. In 0.01 N NaOH, the activity is stable at 23°C for 24h but is lost in 3h at 40°C in 0.1 N NaOH and in 15 min at 90°C, pH 7 - 9 (Matsushima, 1958).

Ficin-papain inhibitor

A third protease-inhibitor was isolated from chicken egg white by Fossum and Whitaker (1968). It inhibits ficin and papain but not trypsin, chymotrypsin or proteolytic enzymes from Pseudomonas aeruginosa, Proteus vulgaris, B. cereus and B. subtilis. This inhibitor can be differentiated from ovomucoid and ovoinhibitor by CM-cellulose chromatography, lack of carbohydrate and molecular weight (less than one half that of ovomucoid). It is extremely heat resistant ; only 10% of its activity is lost after boiling for 30 min at pH 4, however at pH 9 only 40% of the original activity remains. It reacts to form a 1:1 complex with ficin or papain ; there is competition between the two enzymes probably because of common binding sites.

As proteolytic enzymes have not been recovered from egg white, the

biological role of the inhibitors cannot be defined and there is no direct evidence to support the assumption (Ayres and Taylor, 1956) that they have a role in the antimicrobial defence. It has been suggested (Board and Fuller, 1974) that these proteins may inhibit proteases released by spermatazoa that did not take part in fertilization.

TABLE 4

SOME PROPERTIES OF THE MAIN PROTEINS OF HEN ALBUMEN

	Protein	Amount in Albumen (%)	MW	pI	Characteristics
1	Ovalbumin	54	46,000	4.5	Phosphoglycoprotein
2	Ovotransferrin	12	80,000	6.05	Chelation of metal ions particularly iron
3	Ovomucoid	11	28,000	4.1	Inhibition of Trypsin
4	Lysozyme	3.4	14,600	10.7	Hydrolysis of β (1-4) glycosidic bond in peptidoglycans Electrostatic interaction with ovomucin
5	Ovomucin	3.5	ND	4.5-4.7	See 4 above
6	Ovoinhibitor	1.5	44,000-49,000	5.1	Inhibition of several proteases
7	Ovomacroglobulin	0.5	760,000-900,000	4.5	--
8	Ovoglycoprotein	1.0	24,400	3.9	--
9	Ovoflavoprotein	0.8	32,000	4.0	Chelation of riboflavin
10	Avidin	0.05	70,000	9.5	Chelation of biotin

ND Value not determined or reported

TABLE 5

SOME OF THE MORE IMPORTANT INTERACTIONS BETWEEN LYSOZYME AND ITS SUBSTRATE

Amino acid residue	Type of bond	Interaction
ASP 101	H Bond	A Ring amide NH
ASP 101	H Bond	B Ring C6
TRP 62	H Bond	C Ring C_6OH
TRP 62	v.d. Waal	B Ring
TRP 63	H Bond	C Ring C_3OH
ASN 59	Main Chain H Bond	C Ring amide C=O
ALA 107	Main Chain H Bond	C Ring amide N-H

TABLE 6

COMBINING SITE AMINO ACIDS OF AVIAN TRYPSIN INHIBITORS

Inhibitor	Essential amino acid
Chicken ovoinhibitor	Arginine
Chicken ovomucoid	Arginine
Turkey ovoinhibitor	Arginine
Turkey ovomucoid	Lysine
Cassowary ovomucoid	Lysine
Penguin ovomucoid	Lysine
Duck ovomucoid	Lysine
Quail ovoinhibitor	Arginine
Quail ovomucoid	Lysine

TABLE 7

GENERAL SIGNIFICANCE OF IRON IN BIOLOGY

O_2 storage by myoglobin.

O_2 transport by haemoglobin.

Electron transport by cytochromes.

N_2 reduction by ferredoxin and nitrogenase.

N_2 fixation by plant root nodules.

Oxygenation and hydroxylation.

Synthesis of DNA.

Certain flavoproteins, e.g. succinic dehydrogenase.

H_2O_2 decomposition by catalase and peroxidase.

Superoxide decomposition by superoxide dismutase.

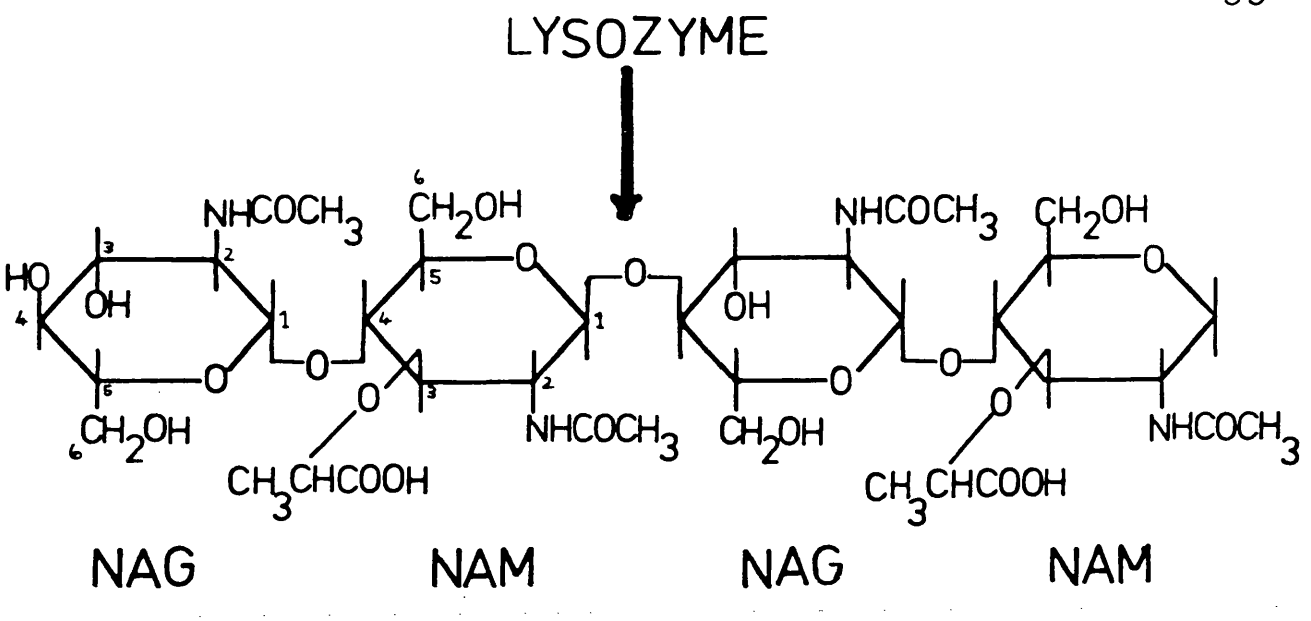


Fig. 1. Schematic diagram of a bacterial cell wall tetrasaccharide, showing the β(1-4) linkage that is attacked by lysozyme. The NAG-NAM bonds are not attacked by lysozyme.

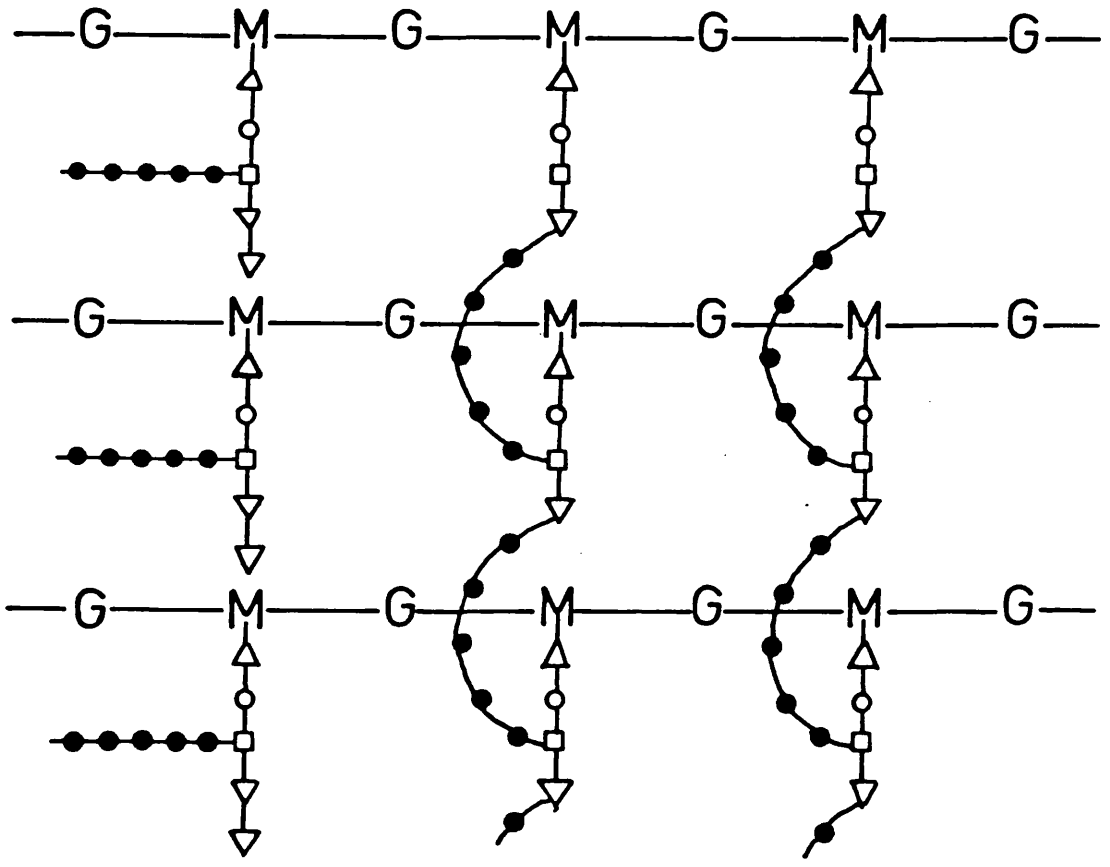


Fig. 2. Schematic diagram of the basic structure of bacterial cell wall peptidoglycan.
G = N-acetylglucosamine ; M = N-acetylmuramic acid ; Δ = L-alanine ; O = D-isoglutamine ; □ = L-lysine ; ∇ = D-alanine ; ● = glycine. The pentaglycine bridges on the left are in amide linkage to lysine but have not yet been connected to the carboxyl of D-alanine. The completed pentaglycine bridges are all shown connecting parallel polysaccharide chains in the plane of the paper. However, they can extend to equivalent peptidoglycan sheets above or below the plane of the paper.

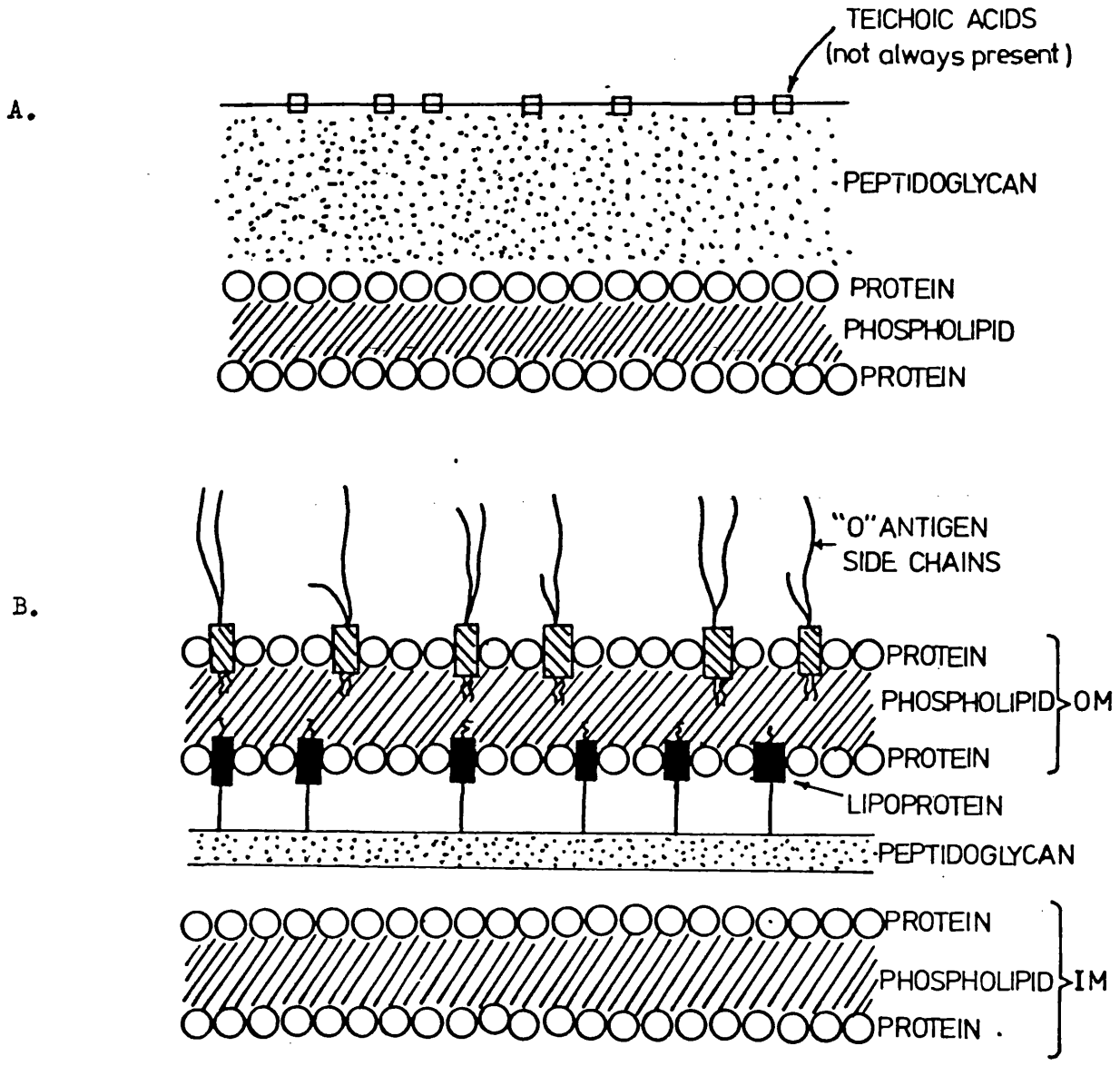


Fig. 3. Schematic representation of bacterial cell wall structure.

A. Gram-positive bacterium in which the trilaminar cytoplasmic membrane (CM) is covered by a thick (200-800Å) layer of peptidoglycan. In some species, teichoic acids may be covalently attached onto the outer surface.

B. Gram-negative bacterium with complex triple layered cell wall. The trilaminar cytoplasmic or inner membrane (IM) is covered by a thin (20-30Å) layer of peptidoglycan and a trilaminar outer membrane (OM), which is attached to the peptidoglycan by a continuous layer of lipoprotein embedded in the former and covalently attached to the latter. The outer membrane also contains lipopolysaccharide which is accessible to antibodies.

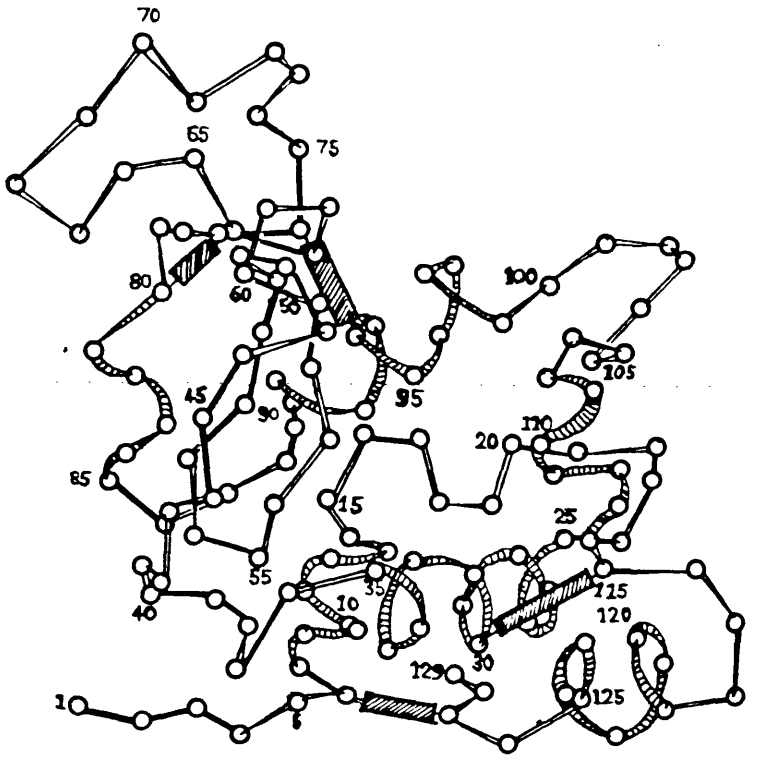


Fig. 4. Schematic representation of the tertiary structure of lysozyme.

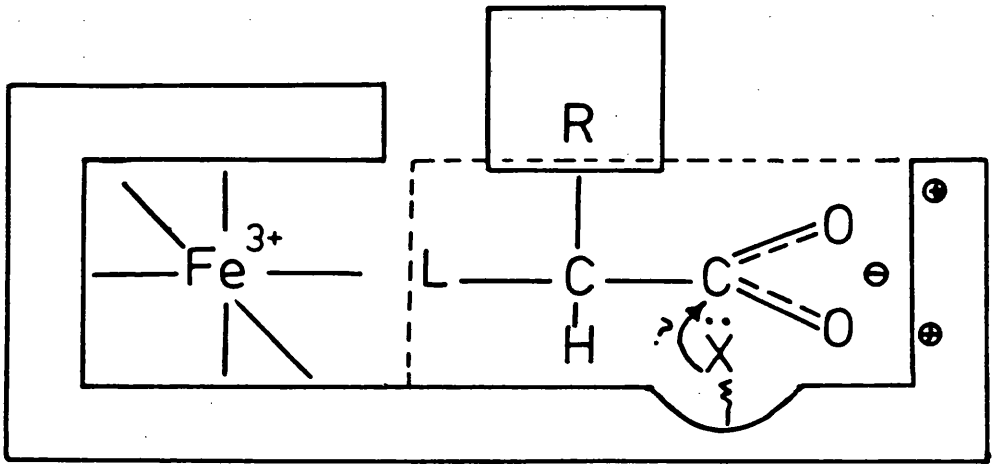


Fig. 5. Schematic diagram of a hypothetical model for the binding of Fe(III) and carbonate to transferrin (from Schlabach & Bates, 1975) : L = proximal ligand co-ordinated to Fe^{3+}; R = large component that projects from the surface of the molecule ; X = protein component possessing a positive charge which makes the carboxy carbon atom susceptible to nucleophilic attack.

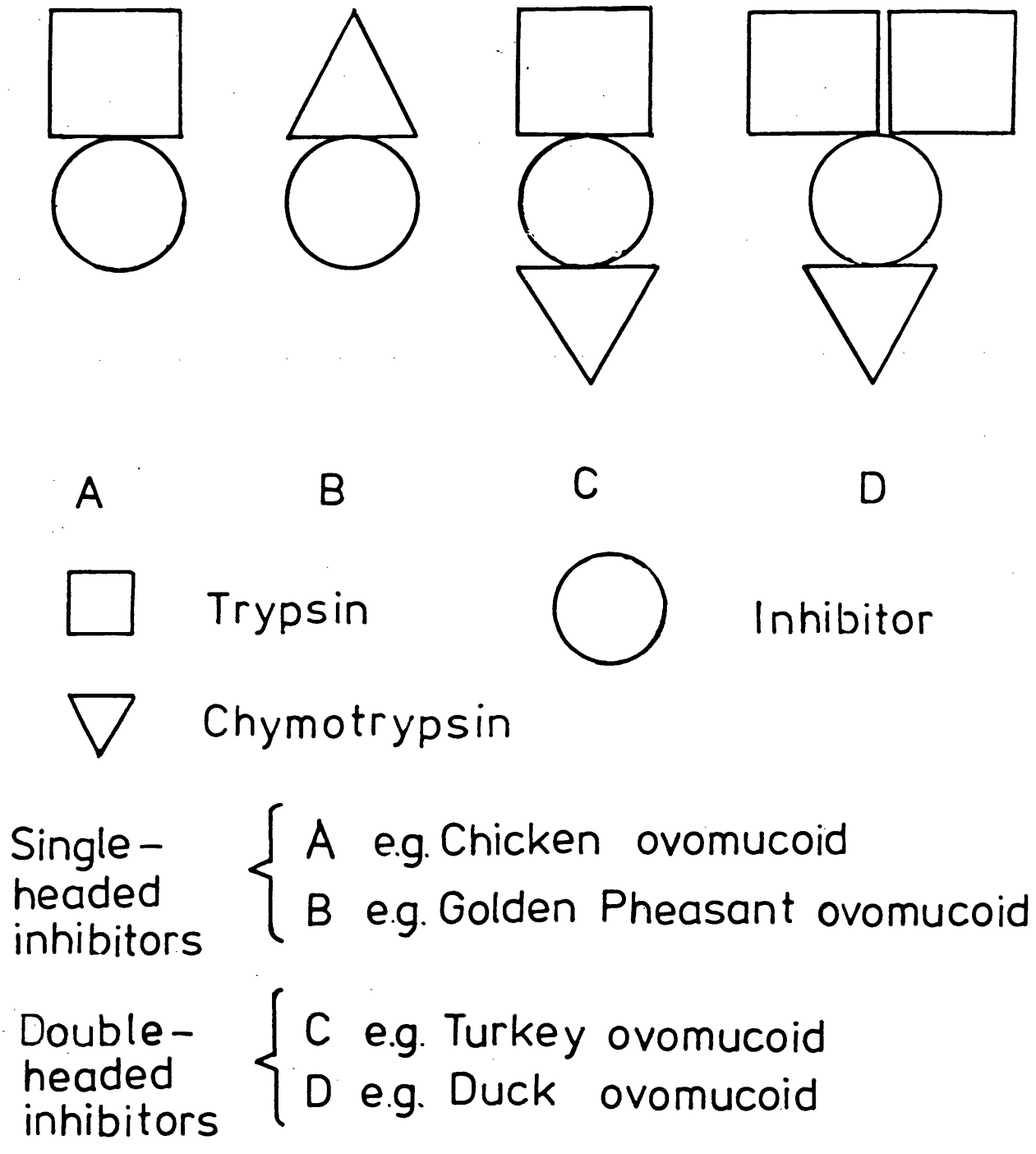


Fig. 6. Schematic diagram of the complexes formed between proteolytic enzymes and avian egg white inhibitors.

IRON TRANSPORT AND METABOLISM IN MICRO-ORGANISMS

Physico-chemical aspects of iron

The contribution of iron to a wide range of biochemical reactions (Table 7) led Neilands (1972) to suggest that "life in any form without iron is in all likelihood impossible". The importance of iron as a bioinorganic catalyst is related to its ability to exist in two stable oxidation states Fe^{2+} and Fe^{3+}. In most biological fluids at or near pH7 and atmospheric oxygen tension, Fe^{2+} will be oxidised to Fe^{3+} which is hydrolysed readily to form insoluble ($K_{SP} < 10^{-38}$ mol/l) high molecular weight polymers of Fe (111) oxyhydroxide (Spiro and Saltman, 1969). The insolubility of these polymers has presumably been an important factor in selecting for organisms that can synthesize compounds which can scavenge and transport Fe^{3+} viz : the siderophores of micro-organisms (Table 8) and the transferrins and ferritins of the vertebrates.

Microbial iron transport

The process of iron assimulation has been studied largely in the enteric bacteria because of the extent of information on their genetics. They possess two distinct iron transport systems (a) a high affinity system consisting of low molecular weight compounds, siderophores, and cognate membrane receptors and (b) a poorly understood, low affinity system which functions in media containing a sufficiently high concentration of readily available Fe^{3+} but no chelate such as N.T.A. Indeed N.T.A. suppresses the second system (Frost and Rosenberg, 1973).

Siderophores

Two classes of siderophores (M. wt 500 - 1000 Daltons) occur : the phenolates (catechols) and the hydroxamates. Although they occur

in bacteria and fungi, the hydroxamates tend to be common in the latter and the catechols in the former. The majority of the siderophores provide six oxygen atoms for engagement with the octahedrally directed bonds of the ferric ion but others, such as the mycobactins (oxygen and nitrogen) pyrimine (all nitrogen) and thioformin (oxygen and sulphur) may possess other electronegative donor atoms. As oxygen atoms are located mainly in the co-ordination sphere, the specificity for Fe^{3+} is high (stability complex 10^{30} mol/l or higher) and Fe^{2+} low.

Catechols

The prototype of the catechol siderophores, a cyclic triester, enterobactin (Pollack and Neilands, 1970) or enterochelin (Cox et al., 1970), occurs throughout most members of the Enterobacteriaceae. Enterobactin (Fig.7) occurs with its hydrolysis products, homologues which can transport iron, albeit less efficiently than the cyclic form (O'Brien et al., 1971). Each enterobactin molecule binds one atom of iron so strongly that Fe^{3+} is difficult to reduce (O'Brien et al., 1971). Enterobactin synthesis (Fig.8) begins at chorismate, the major branch compound in aromatic amino acid biosynthesis ; 2, 3-dihydroxybenzoic acid but not 2, 3-dihydroxybenzoylserine is a key intermediate (Rosenberg and Young, 1974). At least seven genes of E. coli (ent A - ent G) which map at approximately 13 min on the chromosome, are involved (Fig.8) in the biosynthesis from chorismate and serine (Luke and Gibson, 1971 ; Woodrow et al., 1975). Three of the genes ent A, B and G are part of the same operon (Woodrow et al., 1975). Indeed future work may show that the entire ent cluster constitutes a single transcriptional unit. The biosynthesis of enterobactin is strongly repressed by iron (Brot and Goodwin, 1968 ; Young and Gibson, 1969 ; Bryce and Brot, 1971).

Hydroxamates

The ferrichrome family is typical of this class of cyclohexapeptide

siderophores in which the Fe^{3+} is complexed by a tripeptide of acylated δ - N - hydroxyornithine and a second tripeptide of simple amino acids viz. triglycine in the case of ferrichrome. With other members of the family substitutions may occur in the cyclohexapeptide ring or in the acyl group of the hydroxamate linkage (Fig.9). It appears that glycine is an essential residue. The ferrichromes occur in various members of ascomycetes, basidiomycetes and the fungi imperfecti (Neilands, 1972). Although bacteria do synthesize other hydroxamate-containing siderophores e.g. pyoverdine $_{Pf}$ in Ps. fluorescens (Meyer and Hornsperger, 1978), hexapeptides of the ferrichrome type have not been isolated from prokaryotes in general.

Apart from lipid-soluble mycobactin, most siderophores are soluble in polar organic solvents and water. The yield of siderophores, which may range from 10g/l as in Rhodotorulic acid produced from Rhodotorula spp. down to a few mg/l as in most bacteria, may be affected markedly by inorganic and organic constituents of the growth media. A concentration of 10^{-5}g atoms per litre of iron represses siderophore production.

Isolation of the siderophores involves the concentration of growth medium from which the cells have been removed, extraction into an organic solvent and precipitation from a mixture of an alcohol and water (Pollack and Neilands, 1970 ; O'Brien et al., 1971 ; Neilands, 1976 ; Young, 1976). As the iron complexes of the siderophores carry zero (ferrichrome), positive (Ferrioxamine B) or negative (enterobactin) charges, purification can be achieved by chromatography on ion-exchange resins. Chromatography of the complexes is made easier by their characteristic absorption patterns. Ferric-hydroxamates absorb in the region 420 - 440 nm, ferric catechols, for example Fe-enterobactin, absorb maximally at 595 nm.

The membrane system

In order to co-ordinate all six octahedrally-directed valencies of Fe^{3+}, the siderophores must have a size of not less than 600 daltons. This mass prevents diffusion along water-filled pores in the outer membranes of enteric bacteria. Thus, specific transport receptors are necessary. Three systems for iron transport are known : (a) the enterobactin system (Braun et al., 1976) ; (b) the ferrichrome system (Leong and Neilands, 1976 ; Braun et al., 1976) and (c) the citrate system (Frost and Rosenberg, 1973). The first two occur in both S. typhimurium and E. coli but the last one occurs only in the last named organism.

The high affinity iron-uptake system for E. coli is depicted in Fig.10. Competition experiments and mutant studies have shown that ferrichrome shares a receptor with phages T_1, Φ80 and T_5 and colicin M (Fredericq and Smarda, 1970 ; Davies and Reeves, 1975 ; Wayne and Neilands, 1975 ; Hantke and Braun, 1975a), and that enterobactin and colicin B share a common receptor (Guterman, 1973 ; Pugsley and Reeves, 1976a). Polyacrylamide gels have been used to identify these outer membrane receptor proteins with the products of the genes ton A and feu B respectively (Braun et al., 1976 ; Hancock et al., 1976). A third protein, coded for by the gene "cit" (Hancock et al., 1976), has been identified as the receptor for the Fe-citrate complex. These proteins, which are over produced in response to low concentrations of iron (Pugsley and Reeves, 1976b ; Hancock et al., 1976 ; Hollinfield and Neilands, 1978) migrate in the 75 - 85 K region of SDS-polyacrylamide gels. Although specific receptors have been identified little is known about subsequent transport.

A number of mutants of E. coli (fep$^-$) (Cox et al., 1970 ; Langman et al., 1972) are unable to transport Fe-enterobactin across the outer (fep A) and the cytoplasmic membrane (fep B). It is now believed that

fep A strains are identical to the fep B strains described above.

The ton B function is an essential feature of all three high affinity iron-uptake systems. A mutation in this gene not only blocks the three transport systems (Hantke and Braun, 1975b) but also absorption of, and DNA injection from phages T_1 and ϕ 80 (Hancock and Braun, 1976) and killing of the cells by colicins B,I,V and M and the antibiotic albomycin, a structural analogue of ferrichrome (Davies and Reeves, 1975 ; Wayne and Neilands, 1975 ; Hantke and Braun, 1975a). Although the precise function of the ton B gene is unknown, it has been cloned on a plasmid and shown to be a protein with a molecular weight of 35,000 (Plastow and Holland, 1980). It has been suggested that it operates by transporting the substances listed above to the cytoplasmic membrane (Wang and Newton, 1971 ; Frost and Rosenberg, 1975) in energy coupling of the two membranes (Hancock et al., 1977) or in maintaining the proper orientation of the outer membrane receptors with components of the cytoplasmic membrane (Kadner and Bassford, 1978 ; Koninsky, 1979).

Despite the similarity in function and regulation there is a basic difference in the mechanism of intracellular iron release from the complexes of the hydroxamates and enterobactin. The hydroxamates are reused by the cell ; intracellular iron release probably occurs by reduction of Fe^{3+} to Fe^{2+} which is only loosely bound to the siderophore (Cooper et al., 1978). The iron-free ligand is then secreted into the medium. Iron reductases have been reported to release iron from ferrimycobactin (Brown and Ratledge, 1974 ; McCready and Ratledge, 1979) and a siderophore, ferripyochelin from Ps. aeruginosa (Cox, 1980). By contrast, Fe-enterobactin is hydrolysed in E. coli to 2, 3-dihydroxybenzoylserine by a specific esterase (O'Brien et al., 1971). Mutants (Fes B) lacking this enzyme (Langman et al., 1972) are unable to hydrolyse the ligand ; Fe-enterobactin accumulates and cultures of cells turn pink whereas the parent cells remain colourless.

Although the *fes* and *fep* genes map close to the *Ent* genes the former are in a separate operon (Woodrow *et al.*, 1975). One of the problems encountered in the literature concerning the genetics of iron uptake and transport has been that no strict gene notation has been adhered to and hence the same gene may be given different notations. The different genes involved in iron transport are listed in Table 9 together with several other associated genes that have only been partially characterized.

TABLE 8

MICROBIAL SIDEROPHORES

Compound	Ligand System		Source
	Type	Number/mole	
Enterobactin (Enterochelin)	Catechol	3	Aerobacter aerogenes Escherichia coli Salmonella typhimurium Klebsiella pneumoniae Shigella sonnei
2,3-dihydroxy-N-benzoyl-L-serine	Catechol	1	As Above
2,3-dihydroxy-N-benzoylglycine	Catechol	1	Bacillus subtilis
Agrobactin	Catechol Spermidine Threonine	3 1 1	Agrobacterium tumefaciens
Pyochelin	Catechol-like	NA	Pseudomonas aeruginosa
Aerobactin	Hydroxamic acid Carboxylic acid Alcoholic hydroxyl	2 1 1	Aerobacter aerogenes
Schizokinen	Hydroxamic acid Carboxylic acid Alcoholic hydroxyl	2 1 1	Bacillus megaterium
Mycobactins	Hydroxamic acid Phenolic hydroxyl Tertiary N	2 1 1	Mycobacterium smegmatis Mycobacterium tuberculosis Mycobacterium kansasii etc.
Ferrichromes	Hydroxamic acid	3	Aspergillus, Neurospora, Penicillium, Ustilago, Actinomyces, Streptomyces Cryptococcus spp.
Rhodotorulic acid	Hydroxamic acid	2	Rhodotorula, leucosporidium Sporobolomyces spp.
Fusarinines	Hydroxamic acid	1-3	Fusaria, Aspergillus, Gibberella spp.
Ferrioxamines	Hydroxamic acid		Streptomyces, Nocardia spp.
Pyoverdine$_{pf}$	Hydroxamic acid -like	NA	Pseudomonas fluorescens

NA Value not available at the present time.

TABLE 9

GENETIC LOCI LINKED WITH IRON TRANSPORT IN *Escherichia coli* K-12

Locus	Map Position	Characteristics of Mutants
ent A,B,C.	13	Enterobactin biosynthesis ; mutants unable to convert chorismate to DHBA but can take up exogenously supplied enterobactin or DHBA.
ent D,E,F,G.	13	Enterobactin biosynthesis ; mutants unable to convert DHBA to enterobactin but can take up exogenously supplied enterobactin.
aro B.	?	Defective in enterobactin biosynthesis ; only produces enterobactin when supplied with the precursor DHBA.
fes A.	?	A component of enterobactin esterase ; thought to have no effect on the enzyme's activity, but may function in stabilization of the B component.
fes B.	14	B component of enterobactin esterase ; mutants accumulate enterobactin but cannot hydrolyze it to DHBA and thus cannot use Fe- enterobactin as an iron source.
fep A.	13	Resistant to colicins B and D ; defective in transport of Fe- enterobactin across the outer membrane. Missing 81K protein.
fep B.	13	Defective in transport of Fe- enterobactin across the cytoplasmic membrane ; may be a different mutation in the fep A gene.

TABLE 9 (Cont.)

Locus	Map Position	Characteristics of Mutants
cir (*feu A*)	44	Resistant to colicins Ia & Ib ; tolerant to colicin V. Missing 74K protein.
fec A. (*cit*)	6	Defective in Fe-citrate uptake. Gene is co-transducible with *arg F*.
fec B.	6	Defective in Fe-citrate uptake. Gene is not co-transducible with *arg F*.
fhu A.	3	Resistant to bacteriophages T1, T5 & ϕ80 colicin M and albomycin ; specifically blocked in binding and transport of Fe-ferrichrome. Missing 78K protein.
ton B. (*exb A*)	27	Resistant to bacteriophages T1 & ϕ80 and albomycin, tolerance to colicins B,D,G,H,Ia,Ib,M,Q, S1 & V. Chromium sensitive ; hyperexcrete enterobactin, blocked in transport of all chelates.
exb B.	64	Tolerance to colicins B,D,G,H,Ia, Ib,M,Q,S1 & V ; hyperexcrete enterobactin ; methionine auxotrophy ; partially defective in transport of Fe- enterobactin. Chromium resistant. Sensitive to phages T1 and ϕ80.
exb C.	?	Tolerance to colicins B,D,G,H, & M ; hyperexcrete enterobactin ; partially defective in transport of Fe -enterobactin.

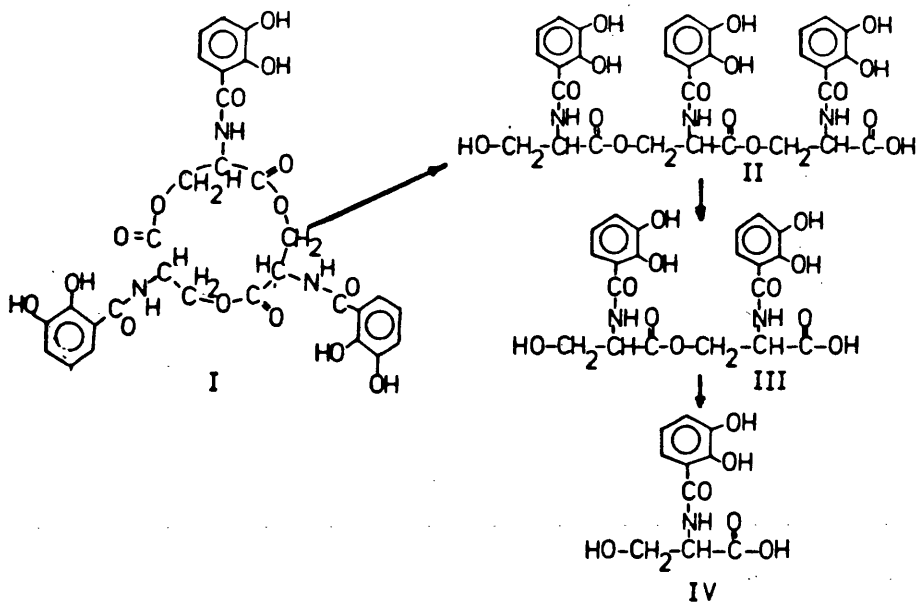


Fig. 7. Enterobactin and its hydrolytic breakdown products :

I. Enterobactin (cyclic DBS trimer).

II. linear DBS trimer.

III. DBS dimer.

IV. DBS (from Neilands, 1974).

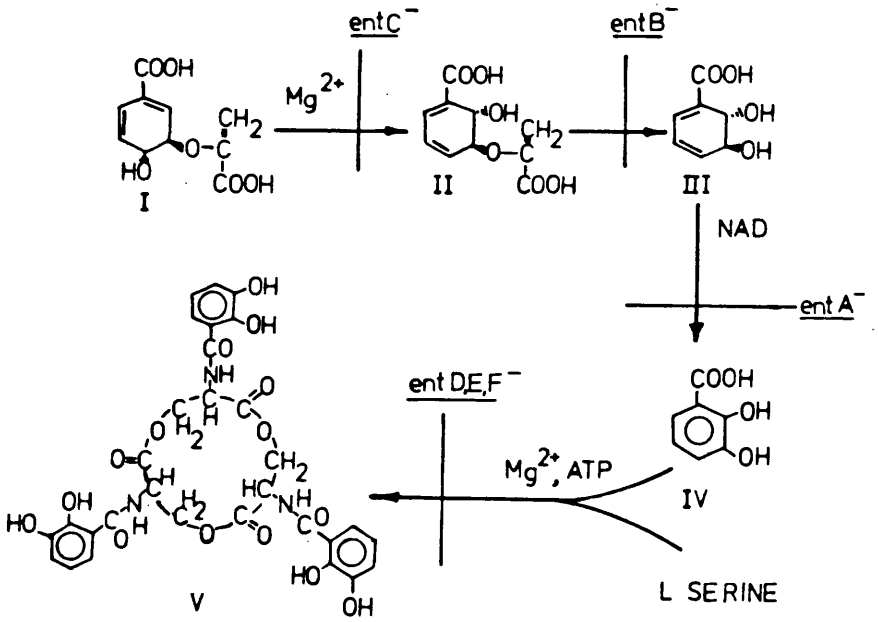


Fig. 8. The biosynthesis of Enterobactin.

I. Chorismate ; II. isochorismate ;

III. 2, 3-dihydro-2,3-dihydroxybenzoic acid ;

IV. DHBA ; V. Enterobactin

The figure shows the reactions affected by mutations in the six ent genes. (from Neilands, 1974).

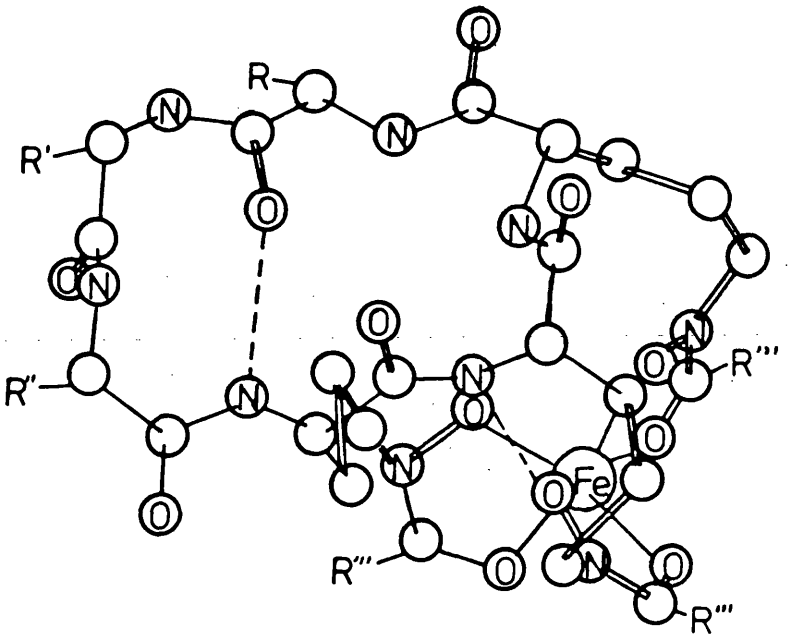


Fig. 9. Hydroxamate siderophores ; the ferrichrome family.

Compound.	Structure.
Ferrichrome.	R=R'=R"=H ; R"'=CH_3-
Ferrichrome A.	R=R'=$HOCH_2$; R"=H;
Ferrichrome C.	R=R"=H R'=R"'=CH_3- (2)
Ferrichrysin.	R=R'=$HOCH_2$-; R"=H; R"'=CH_3-
Ferricrocin.	R=R"=H;R'=CH_2OH; R"'=CH_3-

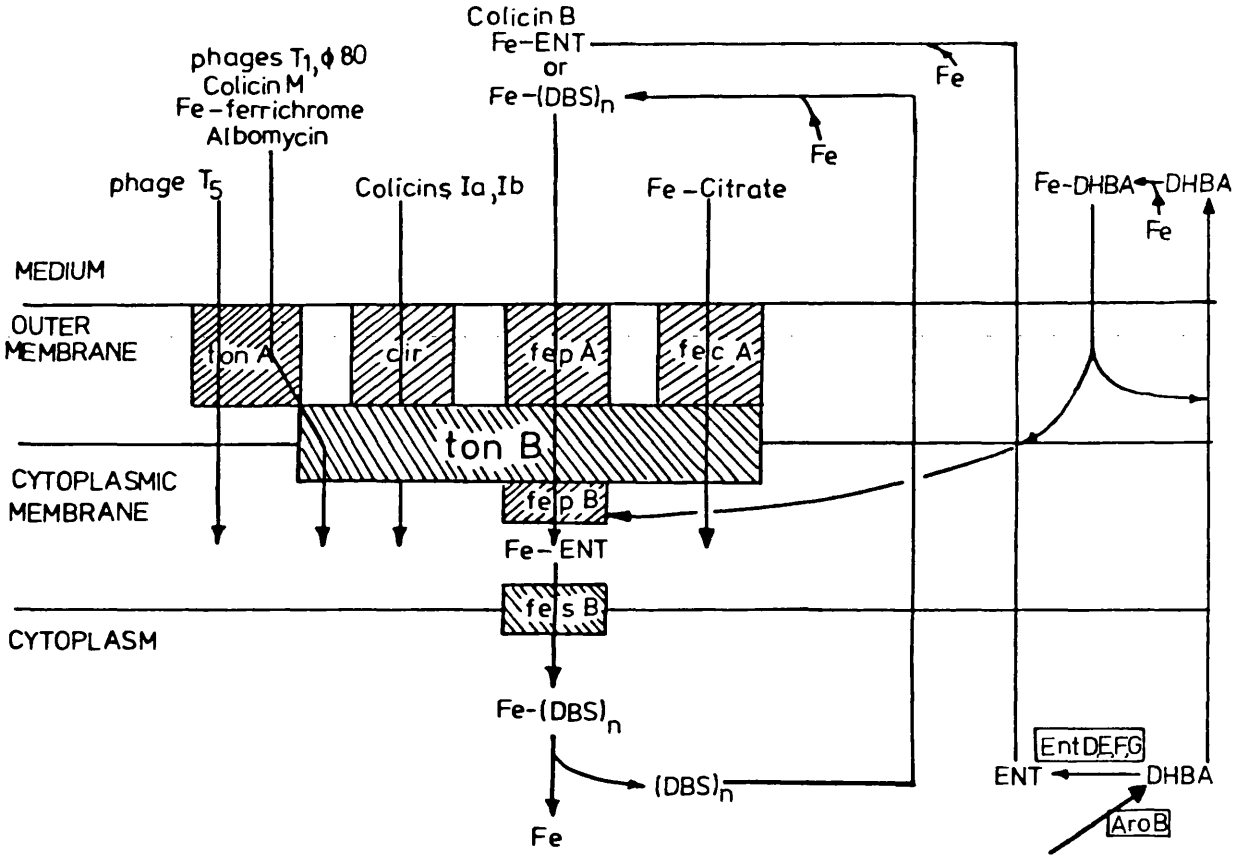


Fig. 10. Receptor-dependent translocation of iron across the outer membrane of Escherichia coli K-12. The figure shows the common binding sites of bacteriophages, colicins and ferri-siderophores in the outer membrane. The proteins coded for by the genes tonA; cir; fepA ; fecA have been identified and located in the outer membrane. Translocation processes requiring the tonB function are shown by an arrow passing through the area designated tonB. Possible pathways for the transport of ferric-enterobactin, ferric-$(DBS)_n$ and ferric-DHBA across the membrane and the release of iron into the cytoplasm are shown. The proposed sites of action of the various gene products are indicated by the inclusion of the gene mnemonic in a box.

MATERIALS AND METHODS

Micro-organisms

The micro-organisms used in this study, together with their maintenance media are listed in Table 10. They were subcultured and their purity checked every four weeks.

Egg supply

(i) Hen eggs

Brown eggs of the domestic hen were obtained from local farm hens housed in batteries and fed on a proprietary diet.

(ii) Duck eggs

Khaki-Campbell duck eggs were obtained from the same local farm as the hen eggs above.

(iii) Turkey eggs

Turkey eggs were obtained from British United Turkeys Ltd., Tarvin, Chester.

(iv) Waterfowl eggs

Eggs of various waterfowl species were collected from nests at the Wildfowl Trust, Slimbridge, Glos.

Albumen

The white of the eggs was harvested aseptically by wiping the eggs with a clean tissue and then swabbing the shell with 70% (v/v) ethanol before cracking the shell with a flamed scalpel and collecting the contents in a sterile Petri dish. Eggs of the waterfowl were candled and those of poor internal quality discarded ; the rest were cleaned with a tissue soaked in distilled water before alcohol sterilisation. The whites from several eggs of the same species were removed from the Petri dish with sterile 10 ml wide-bore pipettes, collected in a sterile screw-capped bottle and mixed by gentle shaking.

Change in albumen pH

When required the pH of hen egg albumen was changed in one of two ways : (a) addition of sterile 1N HCl or (b) passing a sterile gas mixture (5% CO_2 - 10% O_2 - 85% N_2) over the surface of the albumen which was shaken continuously in a 250 ml Erlenmeyer flask.

Hen eggs were placed in liquid paraffin immediately following lay to retard the diffusion of CO_2. This maintained the albumen at about pH 7.5 until it was harvested.

Measurement of pH

The $[H^+]$ of all solutions was measured by pH meter model 10 (Corning Eel Scientific Instruments Ltd.)

Additions to egg albumen

(i) Casamino acids solution

Vitamin-free casamino acids (Difco), deironised with 8-hydroxyquinoline to remove any extraneous iron (Waring and Werkman, 1942) were filter-sterilised (0.45 µm ; Oxoid Ltd.), and added to egg white to give a final concentration of 10 mg/ml.

(ii) Trace element solution

This consisted of (wt/litre distilled water) : NaCl, 0.3 g ; $(NH_4)_2\ SO_4$, 0.66g ; $ZnSO_4 . 7H_2O$, 0.11 mg ; $CaSO_4 . 7H_2O$, 0.11 mg ; $CoSO_4 . 7H_2O$, 0.11 mg ; $MnCl_2 . 4H_2O$, 0.63 mg ; $MgSO_4 . 7H_2O$, 0.14 mg. One millilitre of this filter-sterilised solution (0.45 µm ; Oxoid Ltd.) was added to 25 ml egg white.

(iii) Growth factor solution

This consisted of (mg/litre distilled water) : p-aminobenzoic acid, 10.0 ; folic acid, 1.0 ; cyanocobalamin, 1.0 ; nicotinic acid, 1.0 ; pantothenic acid, 1.0 ; thiamine, 1.0 ; riboflavin, 1.0 ; biotin, 1.0. One millilitre of this filter-sterilised solution (0.45 µm ; Oxoid Ltd.) was added to 25 ml egg white.

(iv) Nitrogen sources

NH_4Cl, $(NH_4)_2 SO_4$, KNO_3 were filter-sterilised (0.45 µm ; Oxoid Ltd.) and added to egg white to give a final concentration of 1 mg/ml.

(v) Germinants

To aid germination of bacterial endospores L-alanine and inosine (final concentrations 10mM and 1mM respectively) were added to egg white.

(vi) Iron transport compounds

Filter-sterilised (0.45 µm ; Oxoid Ltd.) enterobactin and 2,3-dihydroxybenzoic acid were added to egg white to give a final concentration of 10 mg/ml.

(vii) Iron compounds

The following iron compounds were used to titrate pure ovotransferrin and/or were added to egg white to saturate the chelating potential of the ovotransferrin i.e. "one millilitre of albumen binds 20 µg iron" (Theodore and Schade, 1965).

$FeSO_4 . 7H_2O$

$Fe (NH_4)_2 (SO_4)_2 . 6H_2O$

$Fe Cl_3 . 6H_2O$

Fe-nitrilotriacetate (Fe-NTA)

The first two of these compounds included 2.5 ml $10NH_2SO_4$/litre iron solution to prevent hydrolysis of the ferrous iron. Fe-NTA was formed from nitrilotriacetic acid and $FeCl_3$; one millimolar nitrilotriacetic acid was titrated to neutrality with NaOH using methyl red as an indicator and 1mM $FeCl_3$ added to give 1mM Fe-NTA. All iron solutions were filter-sterilised (0.45 µm ; Oxoid Ltd.) and made up fresh each time.

Inoculation of micro-organisms into albumen

Nutrient broth, 'plate count broth' or tryptone soya broth (Oxoid Ltd.) and Y.E.P.G. broth consisting of (g/l) : Yeast extract (Difco), 10.0 ; Peptone (Difco), 20.0 ; D-glucose, 20.0, were used to grow bacteria and yeasts respectively before inoculation into albumen. After incubation for 18h at the temperature stated in the text, the cells were harvested by centrifuging (5,000 x g) and washed twice with sterile quarter strength Ringers buffer pH 7.0 (BDH Ltd.), before finally resuspending them in a solution of the latter.

Portions (25 ml) of albumen, in sterile 100 ml Erlenmeyer flasks were inoculated with 1 ml of a suitably diluted suspension of bacteria or yeast. The inoculum and albumen were mixed by swirling and repeated sucking up and down using a 1 ml wide-bore pipette. The flasks were then incubated in a shaking water bath (60 shakes/min) at the required temperature.

Viable counts

Three methods were used.

(i) The droplet technique (Miles and Misra, 1938) was used for micro-organisms that formed small, discrete colonies on solid nutrient media. Serial decimal dilutions were prepared from 1 ml of albumen in 9 ml of sterile quarter strength Ringers solution. The albumen/Ringers mixture was mixed thoroughly at each step in the dilution series and a different sterile pipette used in each transfer. Eight 0.02 ml drops of each dilution were dispensed onto the dried surface of nutrient agar, plate count agar, tryptone soya agar or M9 minimal agar containing (% w/v) : $Na_2 HPO_4$, 0.53 ; KH_2PO_4, 0.27 ; NaCl, 0.045 ; NH_4Cl, 0.09 ; $CaCl_2 . 2H_2O$, 0.0018 ; $MgSO_4 . 7H_2O$, 0.022 ; D-glucose, 0.35 ; agar, 2.0 ; each component was sterilised separately by autoclaving (121°C/15 min) and added together aseptically just before use. These plates were then

incubated at the optimum growth temperature of the micro-organism concerned and colonies counted after 24h and 48h.

(ii) The plate-dilution frequency technique (Harris and Sommers, 1968) was used for bacteria e.g. Proteus vulgaris, Bacillus cereus T that did not form small discrete colonies on solid media.

(iii) The pour plate technique was used for counts involving yeasts and reconstituted dried baby milk. With the former, the serial dilutions were first sonicated with cooling at an amplitude of 6.0 microns peak to peak on a sonicator (M.S.E. Instruments Ltd.) probe diameter 3mm, before 1.0 ml of appropriate dilutions was used to prepare pour plates with Y.E.P.G.A. which were then maintained at 30°C for 24h and 48h. With the latter 1.0 ml of the appropriate dilution was used to prepare pour plates using plate count agar (3d incubation at 30°C and 37°C ; the general count), MRS agar (deMan et al., 1960) (3d incubation at 30°C and 37°C ; the lactobacilli count) and P.G.Y.E.A. which contained (% w/v) : potato extract (Difco), 0.4 ; D-glucose, 0.25 ; Yeast extract (Difco), 0.4 ; agar, 2.0 (3d incubation at 30°C and 37°C ; the spore count). The spore count was made after heating the reconstituted dried milk at 70°C for 30 min to inactivate the vegetative cells present.

Glassware

All glassware used in the experiments involving iron was freed of contaminating iron by the following procedure : (a) overnight soak in 0.1N HCl (b) rinse with deionised, glass-distilled water (c) wash with 0.5% (w/v) 8-hydroxyquinoline (BDH Ltd.) in chloroform to chelate any iron present (Waring and Werkman, 1942) (d) rinse with chloroform to remove any traces of chelate (e) dry in a hot-air oven to remove chloroform (f) rinse twice with deionised, glass-distilled water (g) dry in hot-air oven. The extraneous iron could be identified by washing untreated glassware with deionised water and determining the amount present which was generally less than 1 µg.

Deironised media

In experiments involving deironised media the method Waring and Werkman (1942) involving extraction with 0.5% (w/v) 8-hydroxyquinoline in chloroform was used to remove contaminating iron.

Chemical determinations

(i) Glucose

Glucose concentrations were determined using the Boehringer blood sugar kit (Boehringer-Mannheim). A sample (0.1 ml) of albumen was precipitated in 1.0 ml 0.16% (w/v) uranyl acetate solution. The protein was pelleted by centrifuging (5,000 x g) and 0.2 ml of the supernatent used for the assay. Five millilitres of glucose oxidase reagent (GOD) consisting of (w/ml) : peroxidase, 20 µg ; glucose oxidase, 180 µg ; chromagen (ABTS), 0.5 mg in 100 mM phosphate buffer (pH 7.0) was added to the supernatant, a glucose standard and a blank of distilled water. The mixture was incubated at 37°C for 20 min and the absorbance of the coloured complex measured at 610 nm by a Unicam SP 500 spectrophotometer (Pye-Unicam Ltd.)

$$\text{Glucose concentration} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100 \text{ (mg/100 ml)}$$

(ii) Iron

Iron concentrations were measured in two ways : (a) using the chromophore bathophenanthroline (4, 7-diphenyl - 1, 10 - phenanthroline) in 0.0025 M in isoamyl alcohol (Peterson, 1953). As bathophenanthroline forms a complex with iron II and not iron III the iron was first reduced from Fe^{3+} to Fe^{2+} with a mixture of trichloroacetic (20% w/v) and thioglycollic (1% v/v) acids, the complex extracted into isoamyl alcohol and its absorbance measured at 535 nm(b) for iron determination in albumen, the protein was first precipitated using the above acid mixture and the supernatant containing the iron analysed by atomic absorption

spectroscopy using the Unicam SP 9 Atomic absorption spectrophotometer (Pye-Unicam Ltd.)

(iii) Catechol siderophores

Total catechol concentration was determined by Arnows (1937) method. The following were added in order to 1.0 ml of sample : 1.0 ml 0.5N HCl ; 1.0 ml Arnows reagent consisting of 10% (w/v) $NaNO_2$ and 10% (w/v) sodium molybdate ; 1.0 ml 1N NaOH and 1.0 ml distilled water. The catechol concentration was determined from a standard curve using 2,3-dihydroxybenzoic acid. Catechols in egg white were determined in the supernatant arising after precipitation of the protein from a 1.0 ml sample with 9.0 ml 0.16% (w/v) uranyl acetate and centrifuging (3,000 x g).

Purification procedures

(i) Ovotransferrin

A combination of the methods of Williams (1962) and Azari and Baugh (1967) was used. The procedure was the same for ovotransferrin from both hen egg albumen and Khaki-Campbell duck egg white.

One litre of egg white was mixed with an equal volume of saturated $(NH_4)_2\ SO_4$ and the precipitated protein removed by centrifuging (10,000 x g). The supernatant was adjusted to pH 4.6 with 0.5$NH_2\ SO_4$ and the precipitate was removed by centrifuging (10,000 x g). Solid $(NH_4)_2\ SO_4$ was added to the supernatant (8g/100 ml) and the ovotransferrin precipitate collected by centrifuging (10,000 x g). The precipitate was dissolved in distilled water and dialysed overnight against distilled water to remove any residual $(NH_4)_2\ SO_4$. The solution, adjusted to pH 6.0 with NH_4OH, was chilled in ice and 0.67 volumes of 50% (v/v) ethanol in 0.02 M NaCl added to precipitate the ovotransferrin which was redissolved in distilled water and dialysed overnight against distilled water. An excess of $FeCl_3 \cdot 6H_2O$ and $NaHCO_3$ was added to form the

orange coloured Fe-ovotransferrin which was then dialysed for 2d against distilled water, adjusted to pH 9.0 with NH_4OH, filtered and the filtrate dialysed against 0.1M ammonium acetate (pH 5.5),

The dialysed filtrate was passed through a column (2.5 x 8.0 cm) of CM Sephadex C25 (Pharmacia Fine Chemicals Ltd.) that had been equilibrated with 0.1 M ammonium acetate. The Fe-ovotransferrin was allowed to run into the surface and top layer of the resin where it was retained as an orange-coloured zone. The column was first washed with 0.1 M ammonium acetate (pH 5.0) and then the Fe-ovotransferrin eluted with 0.1 M ammonium acetate (pH 7.0). Fractions (10 ml) of the eluate were collected and their absorbance determined at 470 nm.

The fractions containing the Fe-ovotransferrin complex were pooled and dialysed overnight against 0.02 M glycine (pH 6.5). They were then passed through a column (2.5 x 5.0 cm) of DEAE Sephadex A50 (Pharmacia Fine Chemicals Ltd.) which had been equilibrated with the same buffer. After washing the Fe-ovotransferrin into the column with more of the same buffer, it was eluted using a gradient formed from 150 ml of 0.02 M glycine in a mixing vessel and 0.02 m glycine - 0.02 MKH_2PO_4 - 0.02 M K_2HPO_4 in an adjacent vessel. Fractions (10 ml) were collected and their absorbance determined at 470 nm.

The Fe-ovotransferrin containing fractions were pooled and the iron dissociated from the protein by adjusting the solution to pH 4.7 with 10% (w/v) citric acid. Stirring for 30 min was adequate to dissociate the iron as the iron-citrate complex. The mixture was then passed down a small column (2.5 x 2.5 cm) of Dowex - 1 anion exchanger (BDH Ltd.) equilibrated with 0.01 M Citrate buffer (pH 4.7) and the protein desalted by gel filtration on a column (2.5 x 30 cm) of Sephadex G25 (Pharmacia Fine Chemicals Ltd.) which had been previously swollen in deionised glass-distilled water. The iron-free protein was eluted with deionised glass-distilled water and the eluate collected in

a pre-weighed glass vessel that had been treated to remove contaminating iron. The solution was freeze-dried and the fluffy white powder weighed. The yield was 4.25g of hen egg ovotransferrin and 1.58g Khaki-Campbell ovotransferrin.

(a) Titration of ovotransferrin with iron

Three millilitres of purified hen ovotransferrin and commercially available hen ovotransferrin (Sigma Ltd.) at a concentration of 2.167 mg/ml in 50 mM borate buffer (pH 9.2) containing 100 mM $NaHCO_3$ were titrated with increasing amounts of iron. After 30 - 60 min to allow colour development the absorbance of the complex was measured at 470 nm (Fraenkel-Conrat and Feeney, 1950).

(ii) Lysozyme

A combination of the methods of Canfield and McMurry (1967), Jollès et al., (1962) and Arnheim et al., (1969) was used.

Pooled hen egg white (300 ml) and 900 ml of 0.05 M NaH_2PO_4 were mixed gently in a Waring blender and filtered through several layers of paper tissue (Kimwipes). CM Sephadex C25 (Pharmacia Fine Chemicals Ltd.) previously equilibrated with the same buffer, was added to the filtrate (4g/l) and the slurry stirred continuously overnight at 4°C. After the resin had settled the supernatant containing the rest of the egg white proteins was decanted and the resin with absorbed lysozyme poured onto a Buchner funnel lined with muslin. The resin was thoroughly washed with an equal volume of distilled water followed by 0.2 M phosphate buffer (pH 6.5). The lysozyme was eluted with 0.6 M phosphate buffer (pH 6.5), dialysed overnight against distilled water at 4°C and freeze-dried.

The freeze-dried sample was dissolved in 10 ml 0.01 M phosphate buffer (pH 5.0). This was applied to the top of a column (2.5 x 25 cm) of CM Sephadex C25 which had been previously washed with 0.1N NaOH, distilled water and 0.1N HCl, soaked in 0.01 M phosphate buffer (pH5.5)

for 48h and then put into fresh buffer and degassed. The column was washed with 0.01 M phosphate buffer (pH 5.5) at a flow rate of 20-30 ml/h. Lysozyme was eluted at a flow rate of 60 ml/h with a buffer gradient produced by placing 350 ml of 0.01 M phosphate buffer (pH 5.5) in a gradient mixing vessel and 0.1 M phosphate buffer (pH 7.5) in an adjacent vessel. Fractions (10 ml) were collected and their absorbance at 280 nm measured. Samples from each peak were assayed for lysozyme activity using the substrate Micrococcus lysodeikticus. The lysozyme-containing fractions were pooled, dialysed against distilled water for 24h at 4°C and freeze-dried.

Lysozyme was desalted by dissolving the freeze-dried sample in 5 ml of 1% (v/v) acetic acid and passing it through a column (2.5 x 25 cm) of Sephadex G-50 (Pharmacia Fine Chemicals Ltd.) which had been equilibrated with 1% (v/v) acetic acid. The lysozyme was eluted from the column with 1% (v/v) acetic acid and fractions (2 ml) collected which were assayed for protein by their absorbance at 280 nm and peak samples assayed for lysozyme activity.

The samples containing lysozyme were pooled, freeze-dried in a pre-weighed glass bottle and stored at -15°C. The yield was 125 mg of hen egg white lysozyme.

(a) Gel electrophoresis of hen egg white lysozyme

The purified sample of hen egg white lysozyme and a commercial preparation of the same (Sigma Ltd.) were subjected to disc electrophoresis (Quickfit Instruments Ltd.) on 15% (w/v) polyacrylamide gel at room temperature. The electrophoresis was run for 2h with a current of 5 mA per tube and 0.001% (w/v) bromophenol blue (BDH Ltd.) added as a marker dye. The gels were stained with Coomassie brilliant blue stain containing 0.1% (w/v) Coomassie brilliant blue ; 50% (v/v) methanol and 70% (v/v) acetic acid and destained by washing (4 x 24h)

in methanol - acetic acid - water (50 : 7 : 43 ; v/v) followed by washing (3 x 24h) in methanol - acetic acid - water (30 : 7: 63 ; v/v).

(b) Lysozyme activity

Bacteria were grown in nutrient broth (Oxoid Ltd.) for 18h at 30°C (Micrococcus luteus) or 37°C (Escherichia coli C20 ; 'Staphylococcus albus' ; Bacillus megaterium). The cells were harvested by centrifuging (5,000 x g) and washed twice in 0.06 M Tris - HCl buffer (pH 9.0) before resuspending in 0.06 M Tris - HCl buffer (pH 9.0) containing 0.05 M NaCl. A freeze-dried suspension of Micrococcus lysodeikticus (Sigma Ltd.) was included for comparison ; 0.1 mg of this substrate was dissolved in 10 ml Tris - HCl buffer containing 0.05 M NaCl. For the assay cells were suspended in the same buffer containing 4 mg/ml hen egg white lysozyme (Sigma Ltd.) and the decrease in optical density measured at 450 nm.

(c) Lysozyme susceptibility of iron-deficient Escherichia coli 0111

Escherichia coli 0111 was grown in M9 minimal medium for 18h at 37°C. The cells were harvested by centrifuging (5,000 x g), washed twice in sterile M9 medium and finally resuspended in sterile M9 medium. One millilitre of this suspension, suitably diluted, was used to inoculate deironised , filter-sterilised (0.45 μm ; Oxoid Ltd.), M9 minimal medium in 0.2 M glycylglycine buffer at the pH stated in the text. Growth of the micro-organism was monitored by the increase in absorbance at 600 nm.

Iron free or iron saturated hen egg white ovotransferrin was added to M9 minimal medium with or without hen egg white lysozyme. All the components were made up in deironised 0.2 M glycylglycine buffer at the pH given in the text, filter-sterilised (0.45 μm ; Oxoid Ltd.) and added together aseptically before inoculation with a suspension of E. coli 0111 as above. In this case growth was monitored by viable counts on M9 minimal medium with 0.05% (w/v) ferric citrate.

(iii) Enterobactin

Enterobactin was prepared from Escherichia coli AN 263 according to the method of Young (1976).

Single colonies of E. coli AN 263 were taken from a culture, grown at 37°C overnight on nutrient agar plates containing 30 mM glucose and 10 mM citrate and streaked across another plate of the same medium to give confluent growth. After 24h at 37°C the growth from this plate was used to inoculate 300 ml of M9 minimal medium containing (final concentrations) : 1.5 mM L-proline ; 0.8 mM L-leucine ; 0.2 mM L-tryptophan ; 1 µM thiamine - HCl and 10 mM citrate. After 22h at 37°C this culture was used to inoculate 10 litres of the same media which had no citrate but additional Fe SO_4. $7H_2O$ (0.2 mM). This culture was grown at 37°C for 22h after which the culture was a deep red because of the Fe-enterobactin present.

The culture was cooled to 4°C and the cells removed by centrifuging (21,000 x g) the medium through a continuous action rotor (M.S.E. Ltd.) fitted to a High Speed 18 Refrigerator Centrifuge (M.S.E. Ltd.) at a flow rate of about 400 ml/min. The resulting supernatant was filtered under vacuum through a glass microfibre filter (G F/D ; Whatman Ltd.) to obtain a clear solution and absorbed onto a column (2.5 x 30 cm) of DEAE cellulose A25 (Pharmacia Fine Chemicals Ltd.) which had previously been equilibrated with 10 mM phosphate buffer (pH 7.0) at 4°C. The Fe-enterobactin was absorbed into the top 2-3 cm of the resin and the column washed with the same buffer. The Fe-enterobactin was eluted with 2 M NH_4 Cl (pH 7.0) at about 250 ml/h and fractions (20 ml) were collected ; brownish-red oxidation products from the medium were left behind on the resin. The red fractions were pooled and the pH adjusted to 1.0 with 10 M H_2SO_4. The acidified solution was extracted (x 3) with ethylacetate (0.1 vol) in a separating funnel and the extracts washed with an equal volume of 0.1 M phosphate buffer (pH 7.0) followed

by 50 ml of deionised glass-distilled water. The aqueous extracts were discarded and the ethylacetate dried over anhydrous Na_2SO_4 overnight. The ethylacetate was concentrated to about 10 ml by rotary evaporation and n-hexane added until the solution was faintly turbid. The ethylacetate - hexane mixture was slowly concentrated by further rotary evaporation until the enterobactin "crystallized". The yield was 40 mg from 10 l of medium.

(a) Non-enzymatic hydrolysis of enterobactin

Enterobactin (10 mg/ml) dissolved in 0.2 M Tris - HCl buffer (pH 9.0 and 7.2) was incubated for 1 h at 37°C, acidified to pH 1.0 with 10 M H_2SO_4 , extracted with an equal volume of ethylacetate and concentrated to approximately 0.5 ml by rotary evaporation. One hundred microlitres of these samples and a control sample were applied to one corner and 1.5 cm from the edge of 20 x 20 cm thin layer Machery Nagel MN 300 cellulose precoated sheets (Camlab - Cambridge) using an Agla Micrometer syringe (Burroughs-Welcome Ltd.) The chromatograms were developed firstly with benzene - acetic acid - water (125 : 72 : 3 ; v/v) inside a fume cupboard, and, after drying at room temperature until the acetic acid was removed, the chromatogram was developed in the second dimension with 5% (w/v) ammonium formate in 0.5% (v/v) formic acid. Enterobactin and its hydrolysis products were detected by ultraviolet light and by spraying with aqueous $FeCl_3$ reagent (O'Brien et al., 1970).

Enterobactin was eluted from the chromatograms in 15% (v/v) acetic acid. The eluate was acidified to pH 1.0 with 10 M H_2SO_4 and extracted into ethylacetate. Hydrolysis products were removed by washing (0.1 M phosphate buffer pH 7.0) and the enterobactin crystallized from the ethylacetate by addition of n-hexane and rotary evaporation.

Bacterial endospores

The materials and methods used in the study of bacterial endospores are listed in the publication bound in the back of this thesis. In addition to these methods those used in the electron microscopy of the endospores are given below.

Electron microscopy of Bacillus cereus T

Spores were removed from egg white and tryptone soya broth by centrifuging (16,000 x g). In each case the pellet of spores was resuspended and fixed in 2% (w/v) $KMnO_4$ at 20°C for 90 min or 5% (w/v) gluteraldehyde in 100mM phosphate buffer pH 7.3 at 4°C for 90 min. The spores suspended in 2% (w/v) $KMnO_4$ were washed at least ten times by repeated centrifugation and resuspension in 100mM phosphate buffer (pH 7.3) until the supernatant was completely clear of residual permanganate. The gluteraldehyde-fixed spores were washed only once in buffer and then both sets of fixed spores were resuspended in 1% (w/v) OsO_4 for 35 min at room temperature in a fume cupboard.

The spores, recovered from the OsO_4 by centrifuging, were washed (x 3) in 100mM phosphate buffer pH 7.3 and dehydrated in 70% (v/v) ethanol for 15 min and then (x 4) in 100% (v/v) ethanol for 15 min. The dehydrated spores were resuspended in a mixture (50 : 50) of 100% (v/v) ethanol and Spurrs resin (Spurr, 1969). The spores and resin mixture was placed in a rotator and accelerator (Taab Ltd.) and after overnight mixing (4 revs/min) at room temperature the spores, recovered by centrifuging (3,000 x g), were embedded in 100% (v/v) Spurrs resin which was polymerised during 48h at 60°C.

Thin sections (< 1 μm thick) of the embedded spores were cut using an ultramicrotome (Reichart, Austria). The sections were stretched by holding a paintbrush soaked in chloroform above them and collected onto

a copper grid. Several grids were stained (saturated uranyl acetate in 70% (v/v) ethanol and Reynolds lead citrate ; 15 min in each) washed with distilled water and dried on filter paper before examination with the JEM 100C electron microscope (Jeol) using an accelerating voltage of 20 kv.

Reconstitution of dried baby milk

Milumil dried modified milk food for babies (Milupa Ltd.) was reconstituted by adding the required amount of powder to sterile 0.2M phosphate buffer or sterile 0.2M borate buffer at 50°C and at the pH stated in the text and mixing to give a final concentration of 10mg/ml. The iron concentration of this milk was 7.0 µg/ml.

TABLE 10

MICRO-ORGANISMS USED IN THIS STUDY

Organism	Maintenance Medium	Source
Acinetobacter sp.	Nutrient Agar	2
Alcaligenes faecalis	Nutrient Agar	1
Bacillus cereus	Tryptone Soya Agar	4
Bacillus cereus T	Tryptone Soya Agar	2
Bacillus megaterium	Nutrient Agar	2
Brocothrix thermosphacta	Nutrient Agar	1
Enterobacter aerogenes	Nutrient Agar	1
Escherichia coli C20	Nutrient Agar	1
Escherichia coli K12 (NCIB 9483)	Nutrient Agar	2
Escherichia coli 0111 *	Nutrient Agar	3
Escherichia coli 0141 *	Nutrient Agar	3
Kurthia zopfii	Nutrient Agar	1
Micrococcus luteus	Nutrient Agar	1
Micrococcus luteus (NCTC 2665)	Nutrient Agar	2
Mycobacterium phlei	Nutrient Agar	1
Proteus vulgaris	Nutrient Agar	1
Pseudomonad sp.	Nutrient Agar	4
Pseudomonas aeruginosa (NCTC 950)	Nutrient Agar	2
Pseudomonas fluorescens	Nutrient Agar	2
Salmonella brandenberg	Nutrient Agar	1
Salmonella dublin	Nutrient Agar	1
Salmonella waycross	Nutrient Agar	1
Serratia marsescens	Nutrient Agar	1
'Staphylococcusalbus'	Nutrient Agar	1
Staphylococcus aureus (NCTC 6571)	Nutrient Agar	2
Streptococcus faecalis (NCIB 8191)	Plate Count Agar	2
Bretanomyces anomalus	Y.E.P.G. Agar	5
Candida krusei	Y.E.P.G. Agar	5
Candida valida	T.E.P.G. Agar	5

TABLE 10 continued

Organism	Maintenance Medium	Source
Debaryomyces hansenii	Y.E.P.G. Agar	5
Saccharomyces cerevisiae	Y.E.P.G. Agar	1

Y.E.P.G. Agar Yeast Extract Peptone Glucose Agar

1 Stock Culture Collection, Bath University Microbiology Dept.

2 Bacteriology Dept., Unilever Limited, Colworth House, Sharnbrook, Bedford.

3 National Institute for Medical Research, Mill Hill, London.

4 Midland Poultry Holdings Limited, The Grove, Craven Arms, Salop.

5 Dr. R.R. Davenport, Long Ashton Research Centre, Long Ashton, Bristol.

* These bacteria were the kind gift of Professor H.J. Rogers.

In addition the following mutant strain was used ; it was maintained on M9 minimal medium with the addition of 1.5mM proline, 0.8mM leucine, 0.2mM tryptophan, 1mM thiamine and 10mM citrate to prevent reversion.

Escherichia coli AN263 FepA 403 proC leu trpE thi rpsl tonA azi strA ara lacY xyl mtl supE.

This culture was the kind gift of Graham Plastow, Dept. of Genetics, Leicester University, Adrian Building, University Road, Leicester.

RESULTS

The literature review identified many proteins of egg white that have antimicrobial properties. The present research dealt with whole albumen so that information on the contribution of any interaction between these proteins could be identified. Particular attention was given to lysozyme and ovotransferrin and the behaviour of bacterial and yeast vegetative cells and bacterial endospores in albumen at different temperatures, iron concentrations and pH. The results of this phase of the work are given in the following sections.

The fate of bacterial vegetative cells in hen egg white

(i) Lysozyme

In a survey (Figs. 11, 12 and 13) of Gram-positive and Gram-negative bacteria, many of which were chosen because they are typical food spoilage organisms, it was found that the former were killed by albumen at 39.5°C the incubation temperature of the hen's egg and a comparative temperature of 30°C irrespective of whether iron was present or not at a concentration sufficient to saturate the white's content of ovotransferrin. Some of the Gram-positive bacteria were not lysed (Fig. 14) by lysozyme purified from hen egg white (Figs. 15 and 16).

Previous workers have demonstrated that Gram-negative bacteria can be rendered susceptible to egg white lysozyme. Incubation under alkaline conditions (Zinder and Arndt, 1956) in the absence or presence of a chelating agent such as E.D.T.A. (Vos, 1964) induces lysozyme-sensitivity in many Gram-negative bacteria. Moreover it is noteworthy that egg white contains several chelating agents (e.g. ovotransferrin, avidin, ovoflavoprotein) and that its pH remains constant at 9.6 within three days of lay (Healey and Peter, 1925).

Escherichia coli 0111 died slowly (Fig. 17) in M9 minimal medium pH 9.0 rendered iron deficient by the presence of ovotransferrin purified from hen egg albumen (Figs. 18a and b), but grew slowly in the same medium at pH 7.4. The addition of lysozyme had no demonstrable effect in either case but the addition of 0.01M$FeSO_4$ resulted in growth in M9 medium at both pH's.

The evidence presented above indicates that lysozyme, although present in considerable concentration in hen egg white, makes only a small if indeed any contribution to the antimicrobial defence of the hen's egg.

(ii) Ovotransferrin

In the past the saturation of ovotransferrin with iron salts in order to allow growth has been the main feature of much of the work concerning the antimicrobial nature of albumen. In some instances the failure of workers to obtain growth of micro-organisms in the presence of "saturated" ovotransferrin may be attributed to the properties of the solutions of the iron salts themselves. It has not been recognized widely in microbiology that ferric salts at neutral pH form polymeric iron complexes, large spherical structures made up of Fe^{3+} cross-linked by oxo-bridges. The release of low molecular weight iron from such polymers is very slow in the absence of a suitable chelating agent such as E.D.T.A., N.T.A. or citrate. Ferrous salts do not exhibit this marked tendency towards hydrolysis and as a result such salts exist as low molecular weight complexes with readily dissociable ligands. Moreover failure to appreciate the chemistry of iron salts at neutral pH can lead to failure in attempts to quench a chelating agent. Indeed the titration of ovotransferrin with ferrous and ferric salts is essential in order to ensure complete saturation

of ovotransferrin before growth experiments involving this protein can be contemplated. This is exemplified by the data presented in Fig. 19. Thus titration of ovotransferrin with ferric salts (e.g. $FeCl_3$) prepared under conditions that did not impede polymer production failed to produce a clear cut end point. The latter was a feature of titrations when steps were taken to prevent polymer formation either by using a ferrous salt prepared in the presence of a small amount of acid or a ferric salt prepared from a suitable chelating agent e.g. N.T.A.

The results obtained when ovotransferrin is less than 100% saturated in hen egg white inoculated with E. coli C3650 are shown in Table 11.

Of the Gram-negative bacteria inoculated into albumen at 30°C (Figs. 11, 12 and 13) Acinetobacter sp. was the only one to die out completely ; most of the other bacterial populations remained static or declined slightly and some populations, notably the Salmonella spp., increased slightly. Acinetobacter was also the only one that failed to multiply when iron was added to albumen at 30°C to saturate the white's content of ovotransferrin.

At 39.5°C, the populations of many more of the bacteria declined in albumen alone (Figs. 11 and 12). With the exception of Ps. aeruginosa none of the other bacterial strains tested was recovered from albumen after 24h incubation. At this temperature iron caused the growth of, or protected the micro-organisms from completely dying out, except in the cases of P. vulgaris, Ps. fluorescens and Acinetobacter sp.

This survey showed that even at an elevated temperature of 39.5°C many of the Gram-negative bacteria which have been associated with the contamination of eggs (Pseudomonas, Escherichia, Salmonella spp.) can

grow providing ovotransferrin is saturated with Fe^{3+}. A knowledge of the physiology of the organisms that did not grow permits a tentative interpretation of their failure. For example, P. vulgaris has complex nutritional requirements that would be unlikely to be satisfied by unsupplemented egg white. Indeed the results presented in Fig. 20 indicate that even with E. coli C20, an organism that has no requirement for vitamins and organic sources of nitrogen, maximal growth and extensive glucose utilisation were features of albumen which contained trace metals, additional combined nitrogen and vitamins, as well as sufficient iron to saturate ovotransferrin.

Several of the bacteria used in this study were examined separately for their response to iron when inoculated into hen egg white. Of several iron salts tested $Fe(NH_4)_2(SO_4)_2.6H_2O$ gave the greatest response in terms of growth of the inoculum when added to egg white (Fig. 21) due possibly to the presence of additional nitrogen (Fig. 22).

The addition of iron sufficient to saturate ovotransferrin had no effect on the inability of Gram-positive bacteria to survive in hen egg white both at 30°C and 39.5°C (Figs. 23-26). Recovery of viable cells was impossible 5-6h after inoculation into hen egg albumen even in the case of a lysozyme-resistant strain such as B. cereus T. There was no evidence of recovery even when the cultures had been left for 24h.

The saturation of ovotransferrin with Fe^{3+} resulted in the growth of Gram-negative bacteria inoculated into hen egg albumen (Figs. 27-31). With the exception of Enterobacter aerogenes, an organism which prefers temperatures around 30°C, all the Gram-negative bacteria grew in the presence of iron at 39.5°C, but were killed in its absence. The first evidence that temperature may influence the fate of bacteria in egg white appeared in the results obtained from the inoculation of Ent.

aerogenes and S. dublin into hen egg white (Figs. 30 and 31). At 30°C, bacteriostasis (Ent. aerogenes) or slight growth (S. dublin) occurred which increased on the addition of iron.

These individual investigations support the results obtained from the general survey of bacteria in hen egg white. The addition of iron to saturate hen ovotransferrin and the influence of temperature on the survival of Gram-negative bacteria only serve to underline the difference between the aforementioned organisms and Gram-positive bacteria in hen egg white.

(iii) Hatchery isolated strains

Hatchery isolated strains of bacteria differed from similar strains obtained from the dept. stock culture collection (grown under identical conditions) when inoculated into hen egg albumen (Figs. 32 and 33). Although the hatchery Pseudononad sp. survived longer in hen egg albumen at 39.5°C it was killed completely after 24h whereas the hatchery-isolated B. cereus was not killed completely at this temperature. At 30°C the hatchery-isolated strains survived significantly better than the dept. stock strains and one of them (the pseudomonad) grew at this temperature indicating that these organisms isolated from the hatchery may possess some factor(s) allowing them to survive longer in albumen and which may ultimately be responsible for problems encountered in the hatching industry.

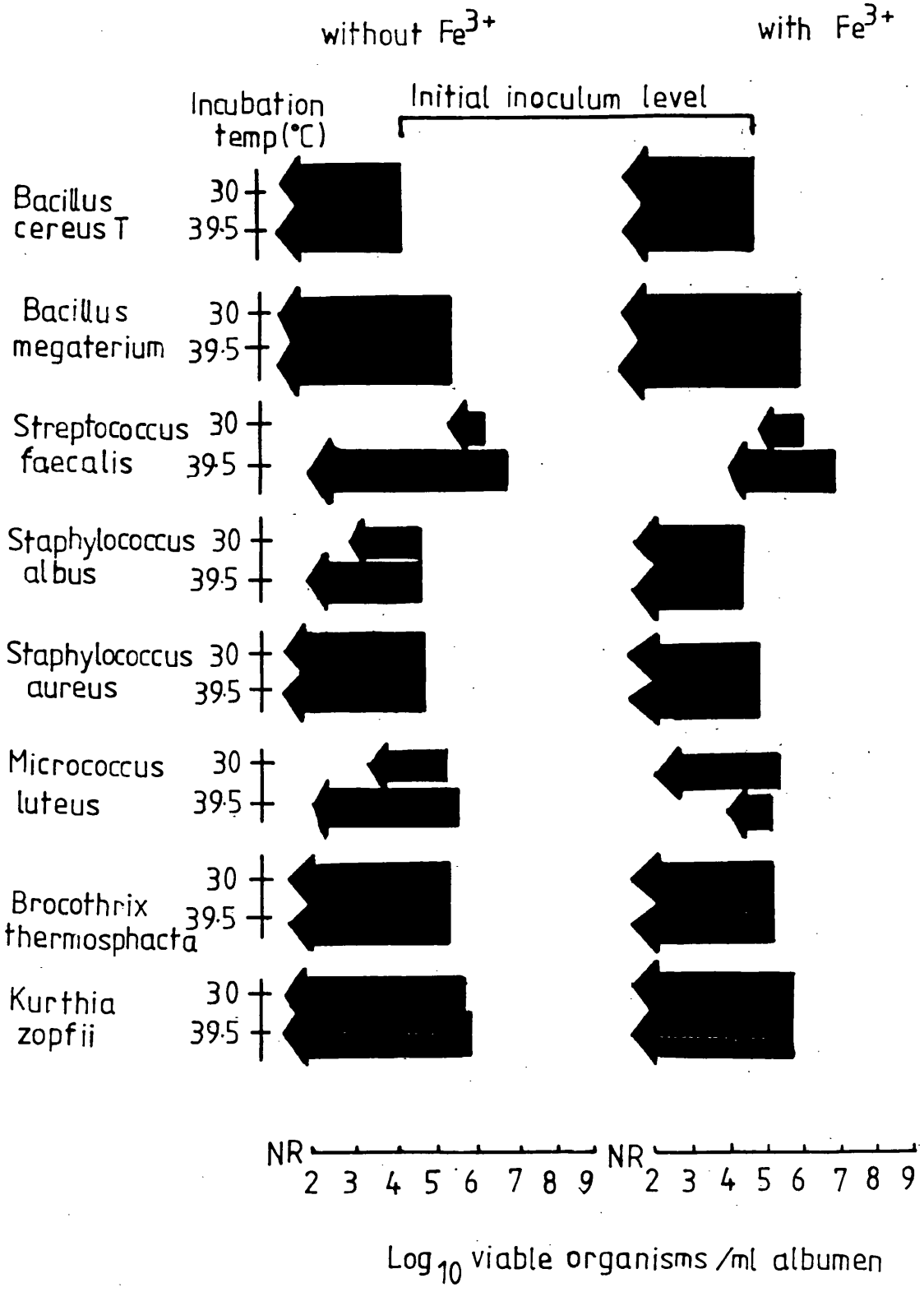


Fig. 11. The effect of iron on the survival of a range of Gram-positive bacteria in hen egg albumen held at 30° or 39.5°C for 24h. NR, viable organisms not recovered.

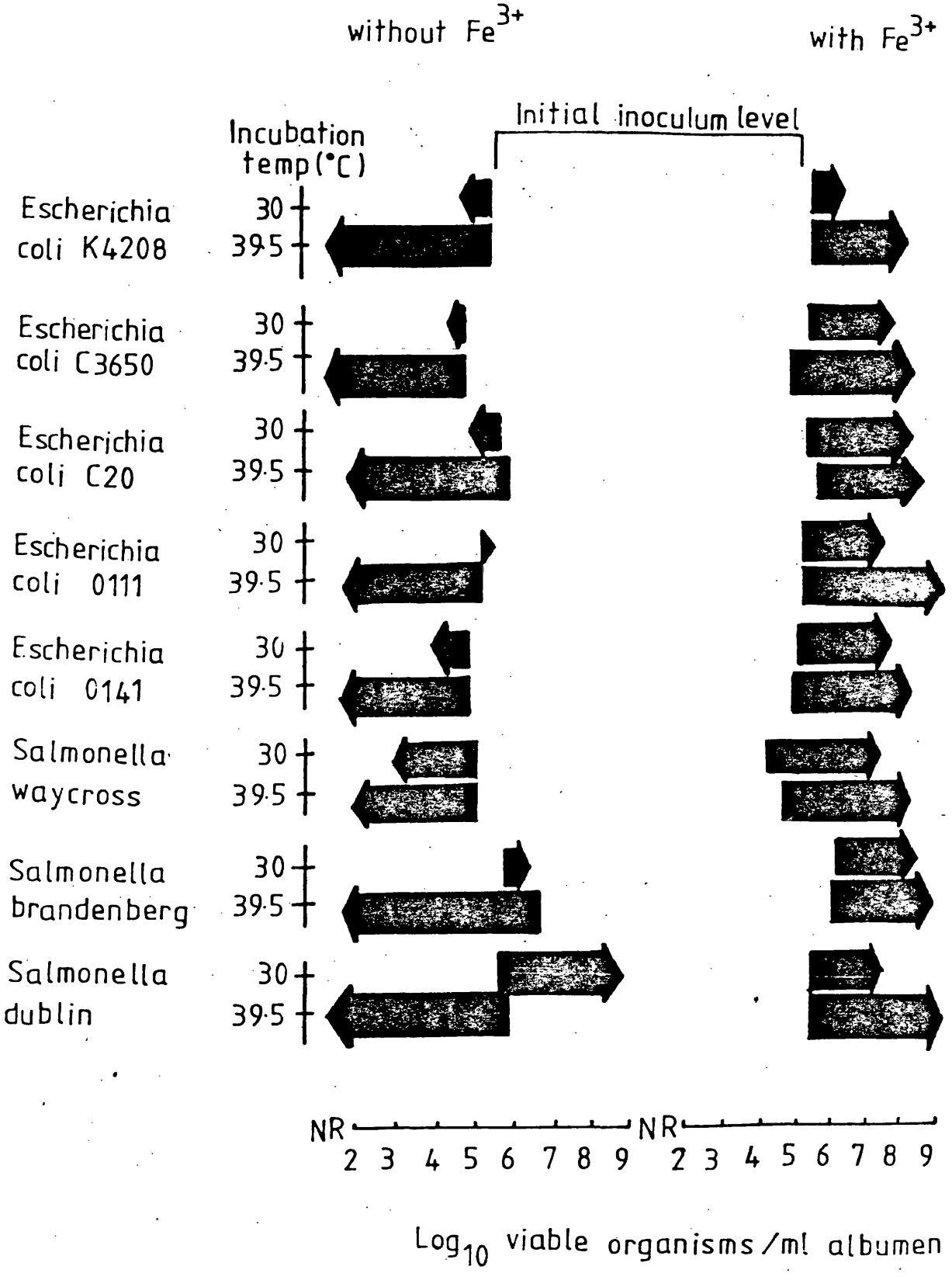


Fig. 12. The effect of iron on the survival of a range of Gram-negative bacteria in hen egg white held at 30° or 39.5°C for 24h. NR, viable organisms not recovered.

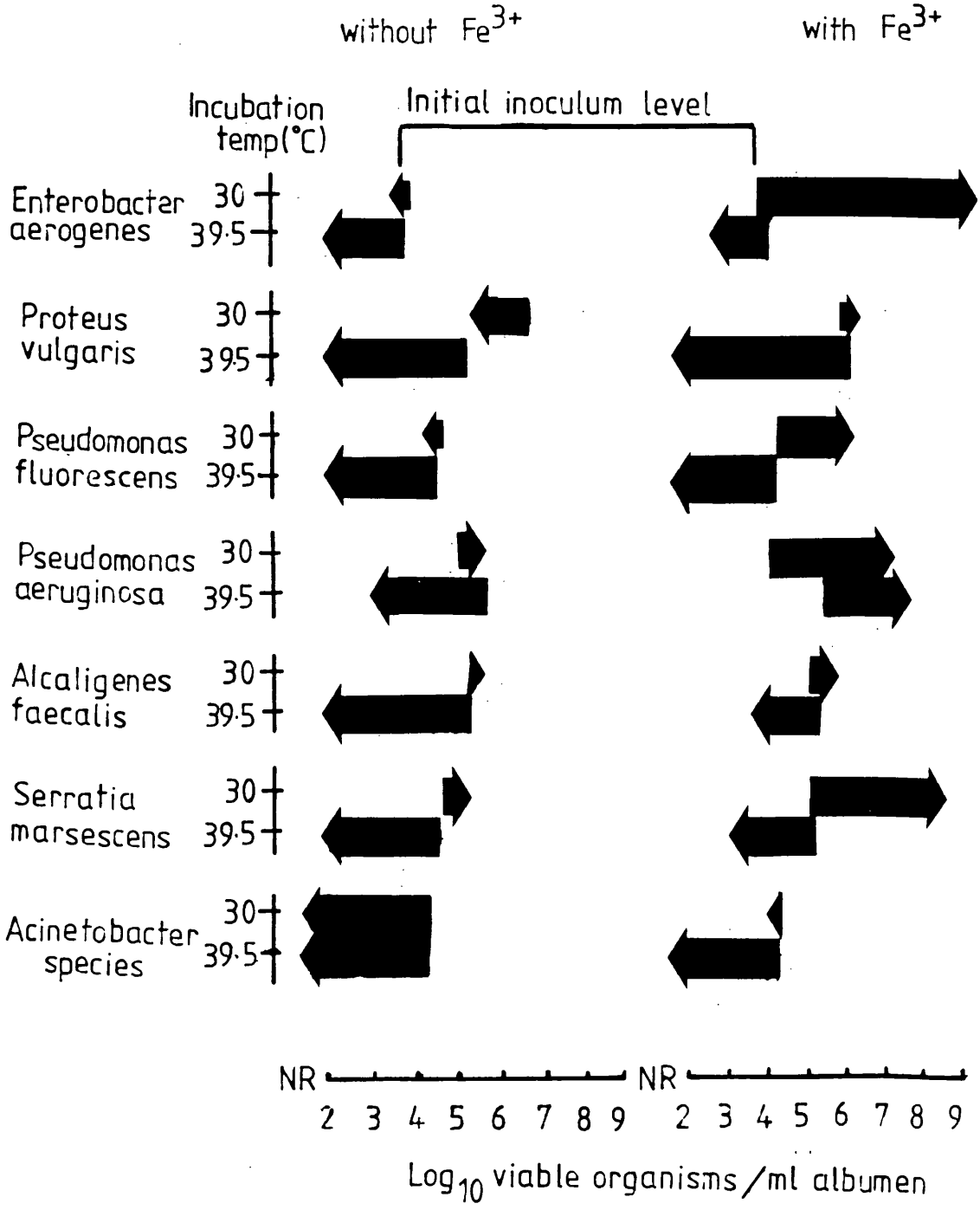


Fig. 13. The effect of iron on the survival of a range of Gram-negative bacteria in hen egg albumen held at 30° or 39.5°C for 24h. NR, viable organisms not recovered.

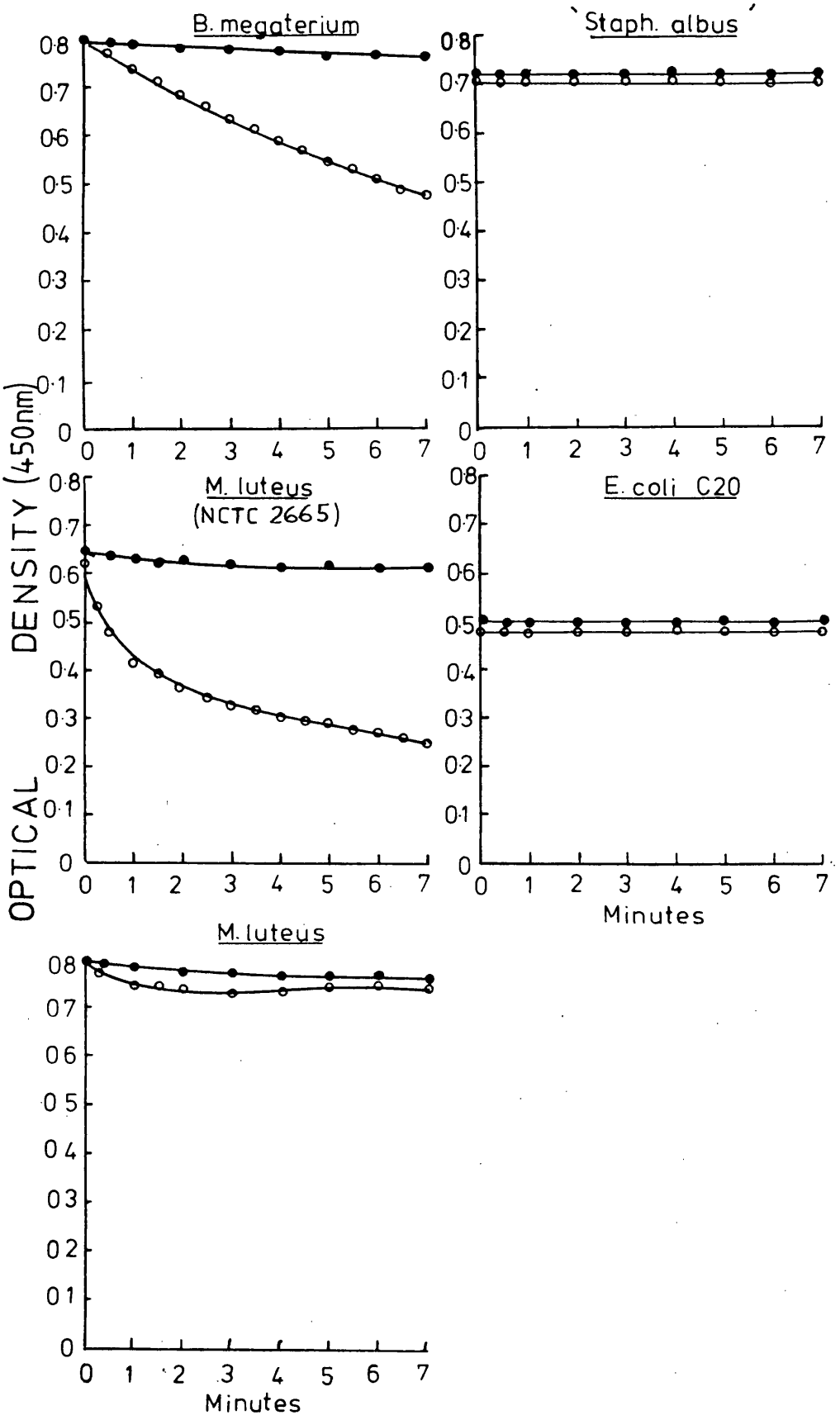


Fig. 14. The action of hen egg white lysozyme on bacteria. A washed suspension of cells was suspended in 0.06M Tris HCl buffer pH 9.0 containing 0.05M NaCl (•) or buffer containing 0.05M NaCl and 4mgml^{-1} lysozyme (o). The decrease in optical density was

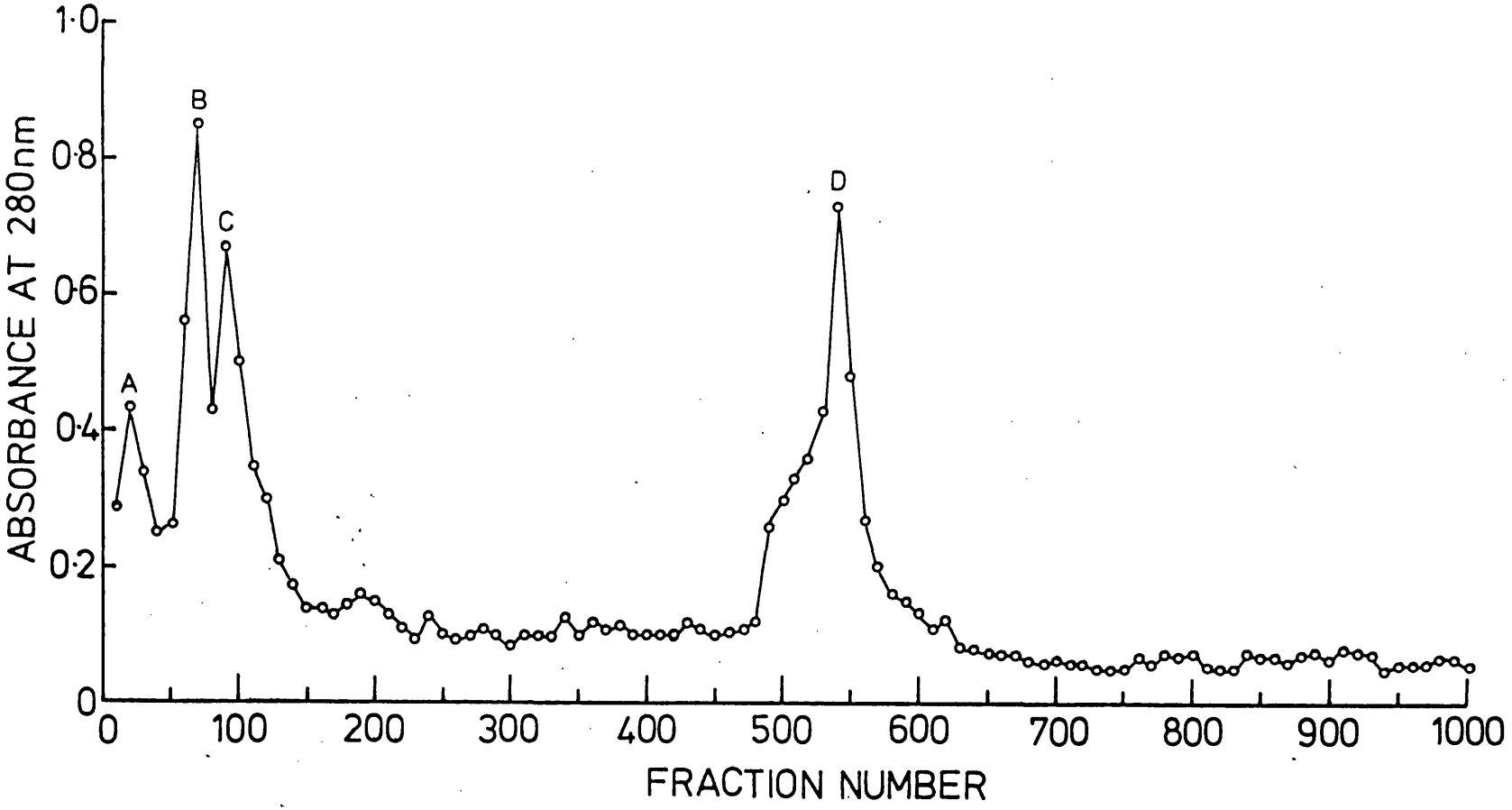


Fig. 15. Chromatography of hen egg white lysozyme on CM- Cellulose. The crude preparation was applied to a column (25 x 2.5cm) which had been equilibrated with 0.01M phosphate buffer pH 5.5. After washing with the same buffer (500ml), the column was eluted with a buffer gradient of phosphate buffer (0.01-0.1M; pH 5.5-7.5); ten millilitre fractions were collected and their absorbance measured at 280nm. When samples from each peak were assayed for their ability to lyse a suspension of Micrococcus lysodeikticus cells only D showed any activity.

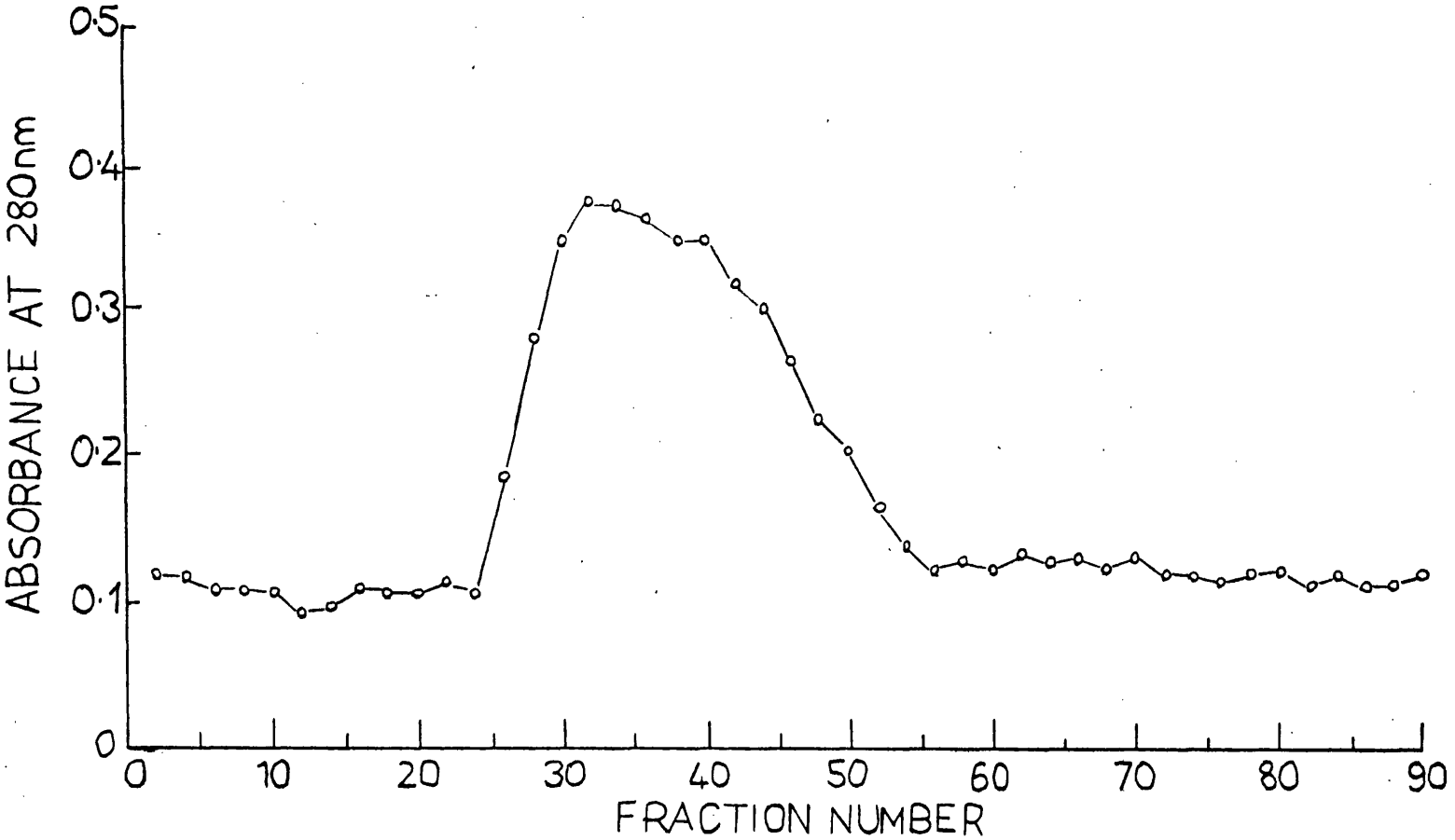


Fig. 16. Chromatography of hen egg white lysozyme on Sephadex G50. The enzyme preparation was applied to a column (25 x 2.5cm) which had been equilibrated with 1% (v/v) acetic acid. The column was eluted with 1% acetic acid; two millilitre fractions were collected and their absorbance measured at 280nm. Samples from the peak showed lytic activity when tested against a suspension of Micrococcus lysodeikticus and gave a single band when applied to a polyacrylamide gel.

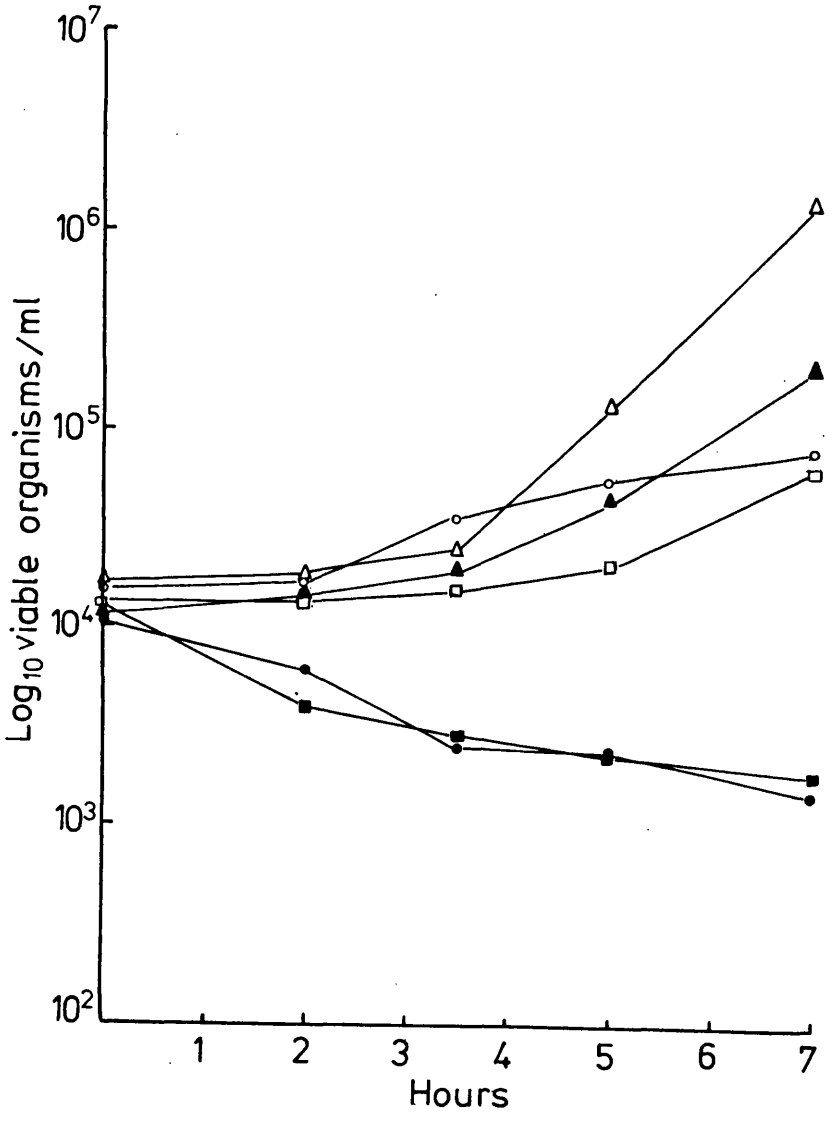


Fig. 17. The effect of 0.01M $FeSO_4$ (△, ▲) and 4mgml^{-1} lysozyme (□, ■) on the fate of Escherichia coli 0111 in M9 minimal medium pH7 (open symbols) and pH9 (closed symbols) in the presence of 1.26mgml^{-1} purified hen ovotransferrin (o , •).

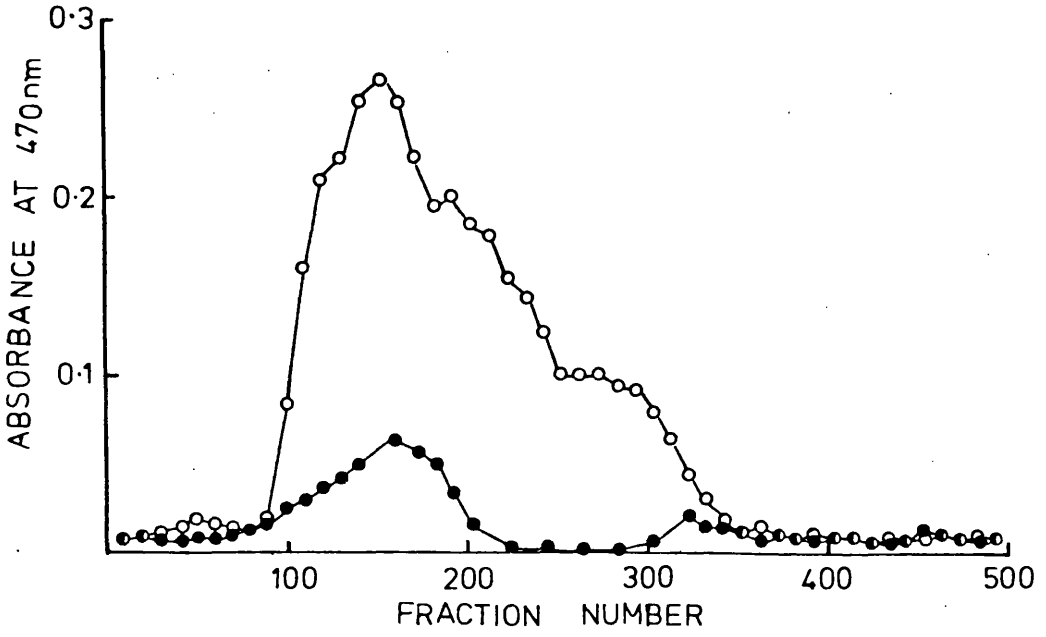


Fig. 18a. Chromatography of hen and duck ovotransferrin on CM-cellulose (see text). The column was eluted with 0.1M CH_3COONH_4 pH7.0 ; ten millilitre fractions were collected and their absorbance (o, hen; •, duck) measured at 470 nm.

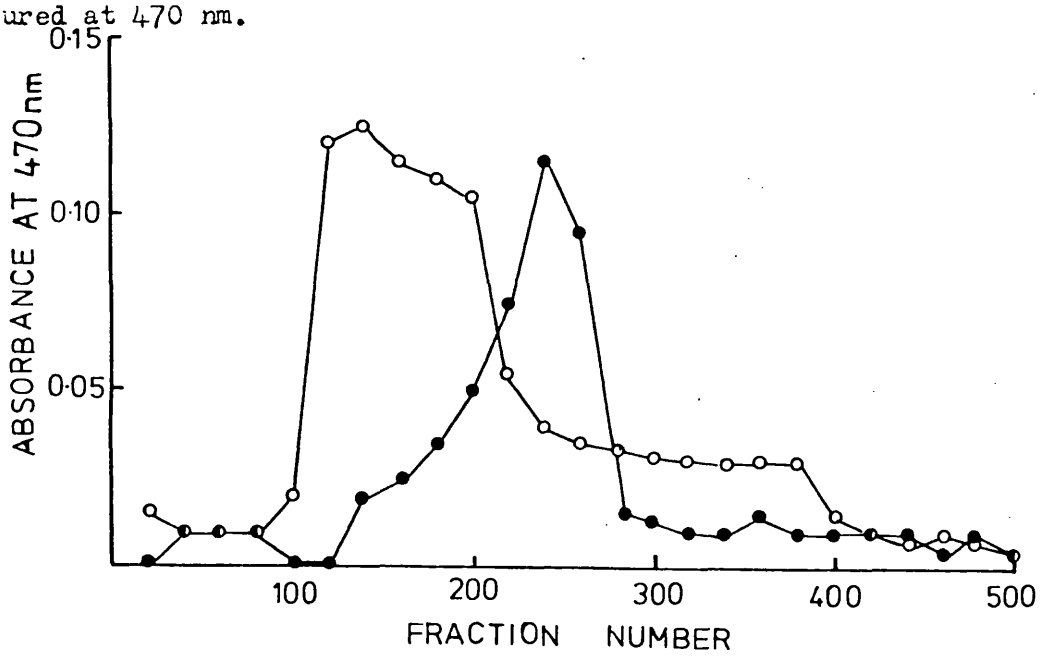


Fig. 18b. Chromatography of hen and duck ovotransferrin on DEAE Sephadex A50 (see text). The column was eluted with a buffer gradient formed from 0.02M clycine-0.02M KH_2PS_4-0.02M K_2HPO_4 ; ten millilitre fractions were collected and their absorbance measured (o, hen; •, duck) at 470nm.

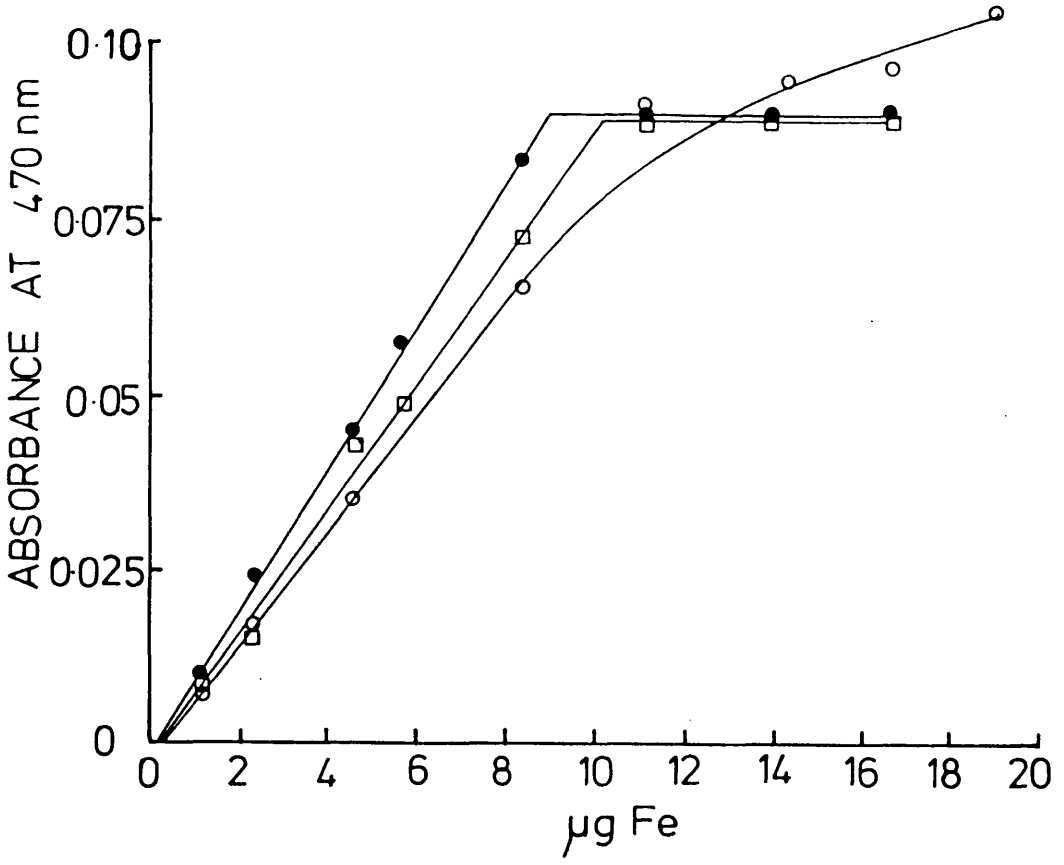


Fig. 19. The spectrophotometric titration of hen ovotransferrin ($2.167 mgml^{-1}$) in 50mM borate buffer pH9.2 containing 100mM $NaHCO_3$ with the iron salts $FeCl_3$ (o), FeNTA (□), and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (•).

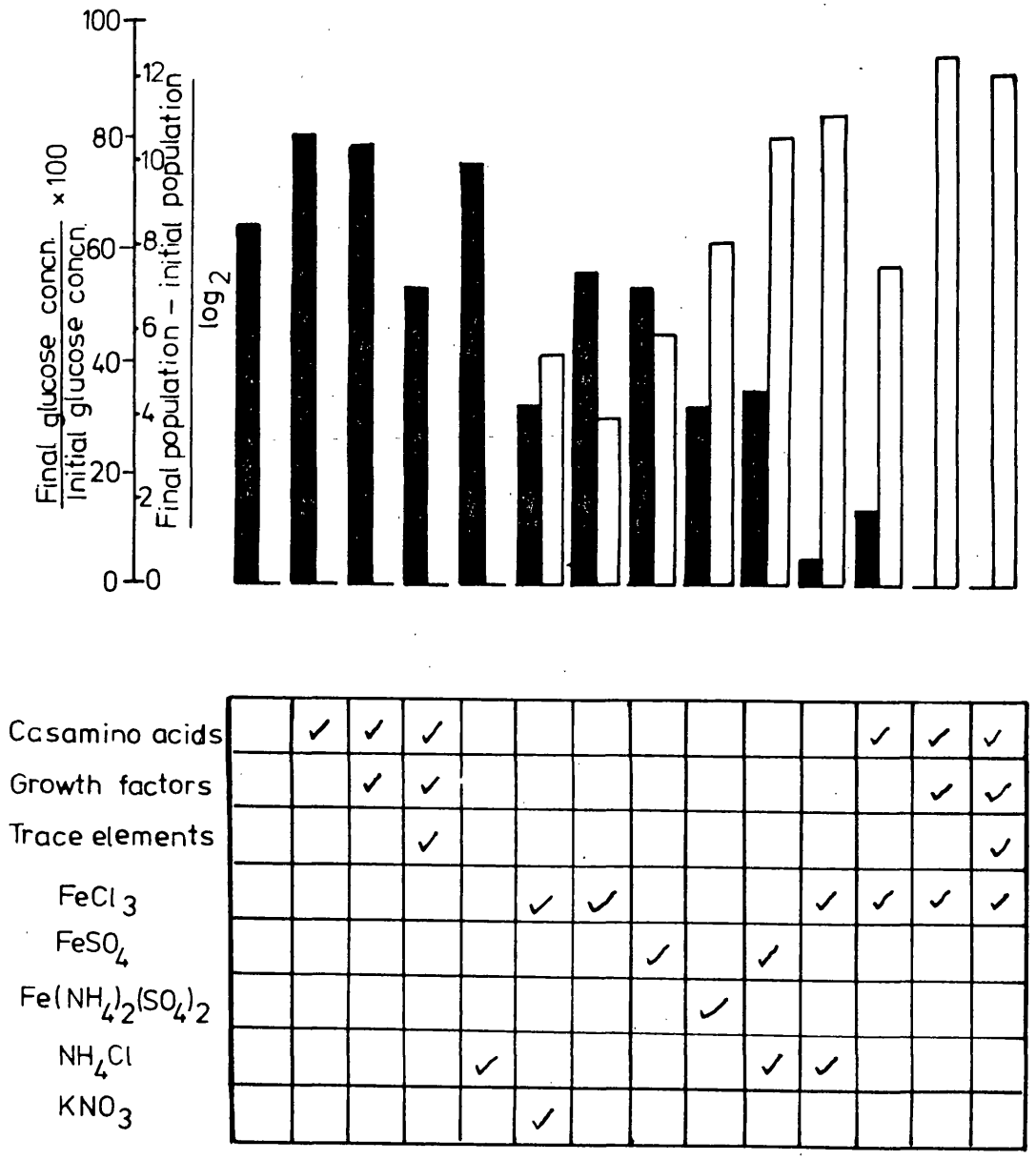


Fig. 20. The effect of various supplements on the fate of Escherichia coli C20 in egg white at 39.5°C.

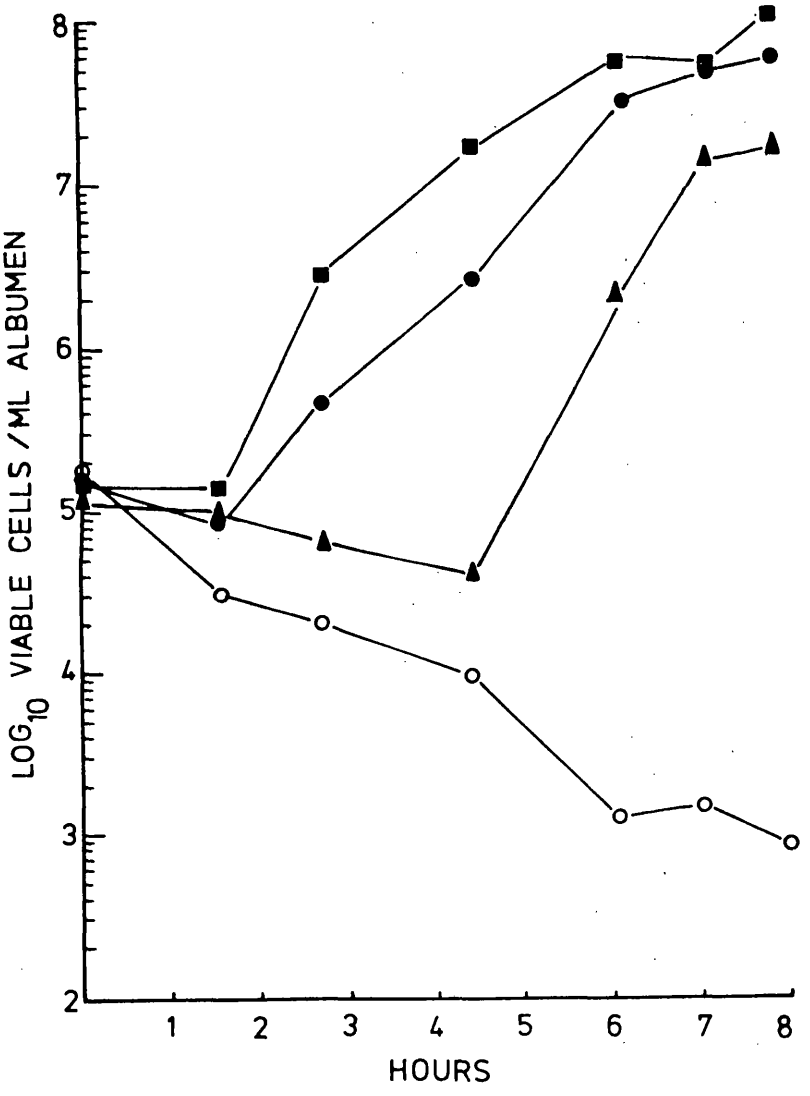


Fig. 21. The effect of iron salts on the death of Escherichia coli 0141 in hen egg white; ▲, $FeCl_3$, ●, $FeSO_4$, ■, $Fe(NH_4)_2(SO_4)_2 . 6H_2O$, o, albumen alone.

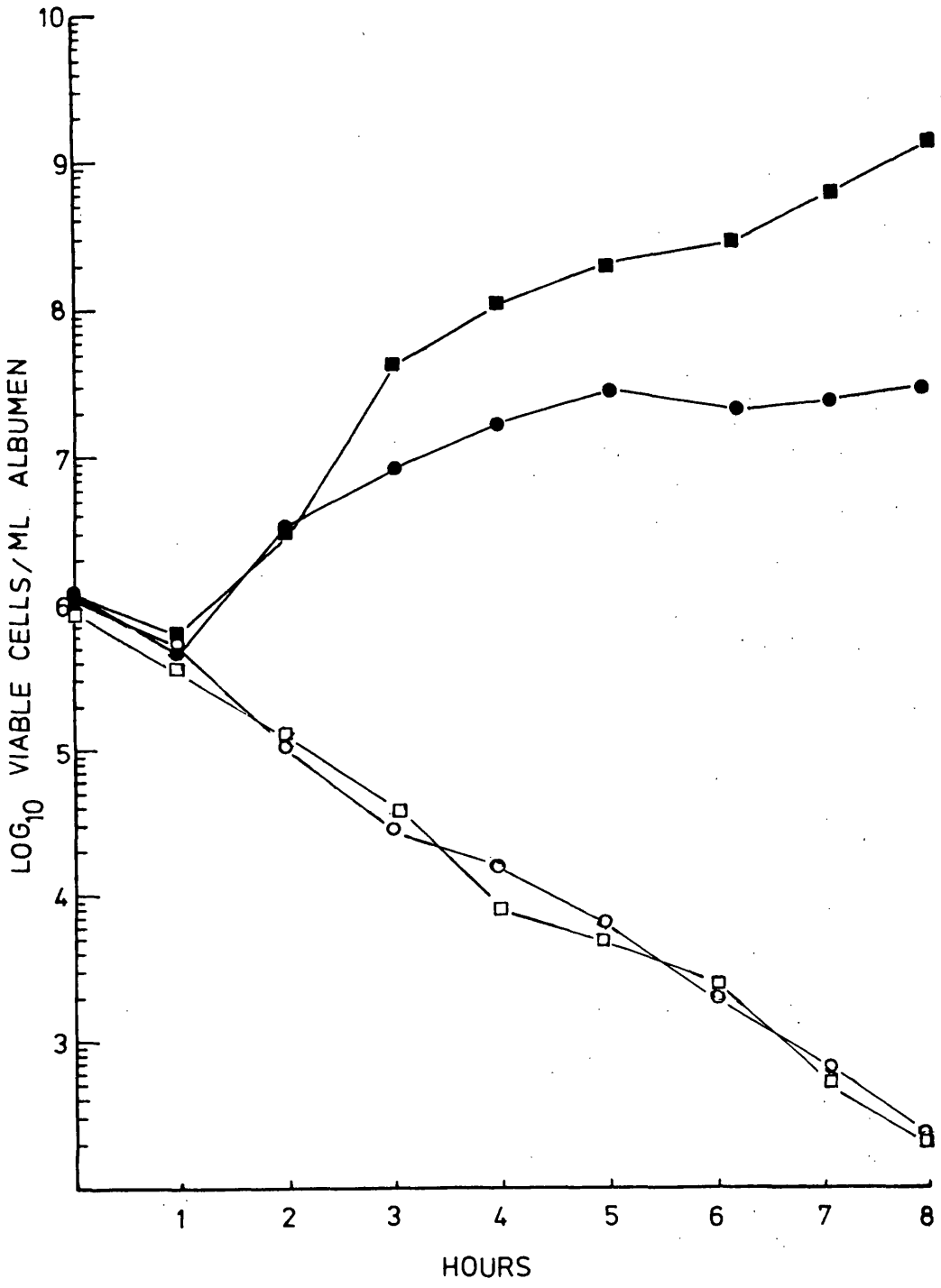


Fig. 22. The effect of casamino acids ($1 mg ml^{-1}$; □ , ■) on the fate of Escherichia coli C20 in hen egg white (open symbols) and egg white saturated with $FeSO_4 . 7H_2O$ (closed symbols) at 39.5°C.

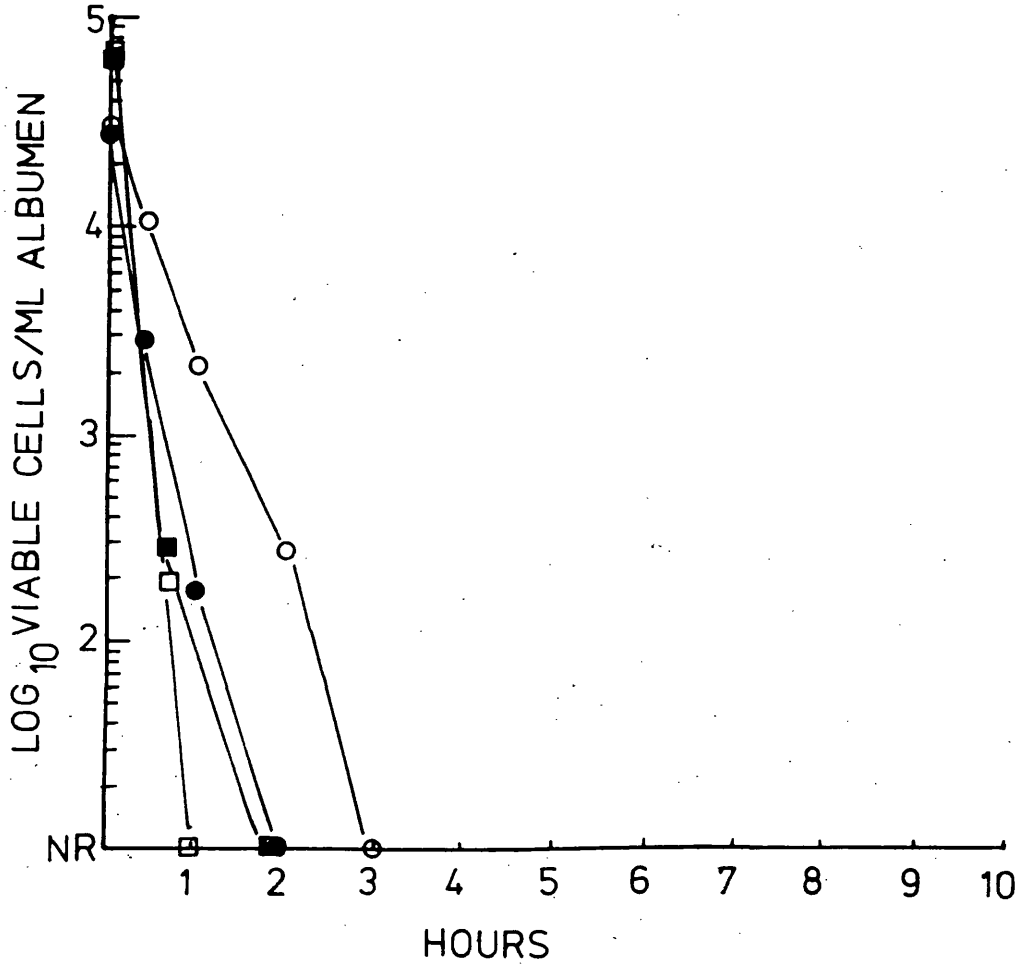


Fig. 23. The fate of Bacillus cereus T in hen egg white (open symbols) and hen egg white saturated with iron (closed symbols) at 30°C (o, •) and 39.5°C (□ , ■).

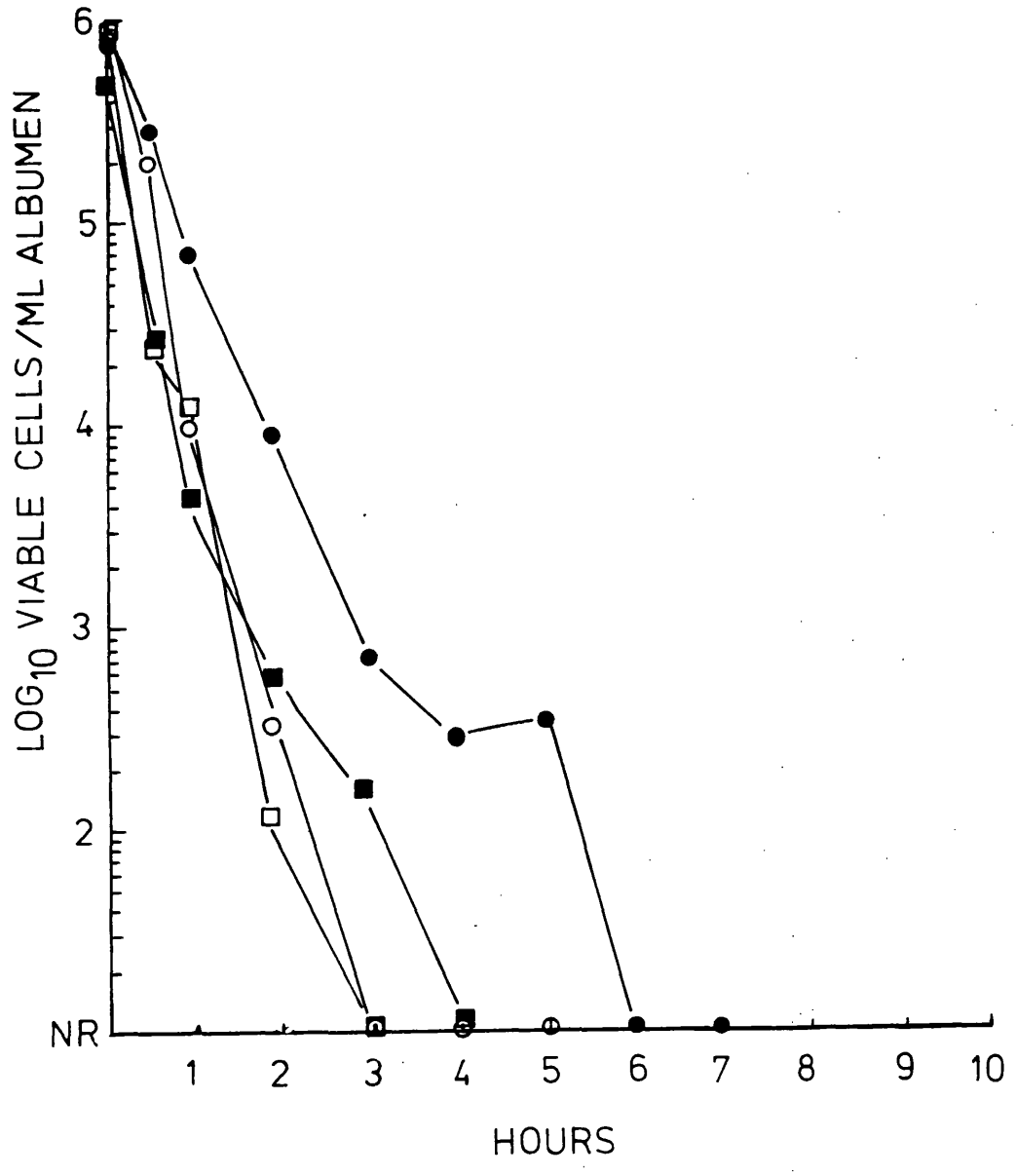


Fig. 24. The fate of Staphylococcus epidermis in hen egg white (open symbols) and iron saturated egg white (closed symbols) at 30°C (o, •) and 39.5°C (□,■).

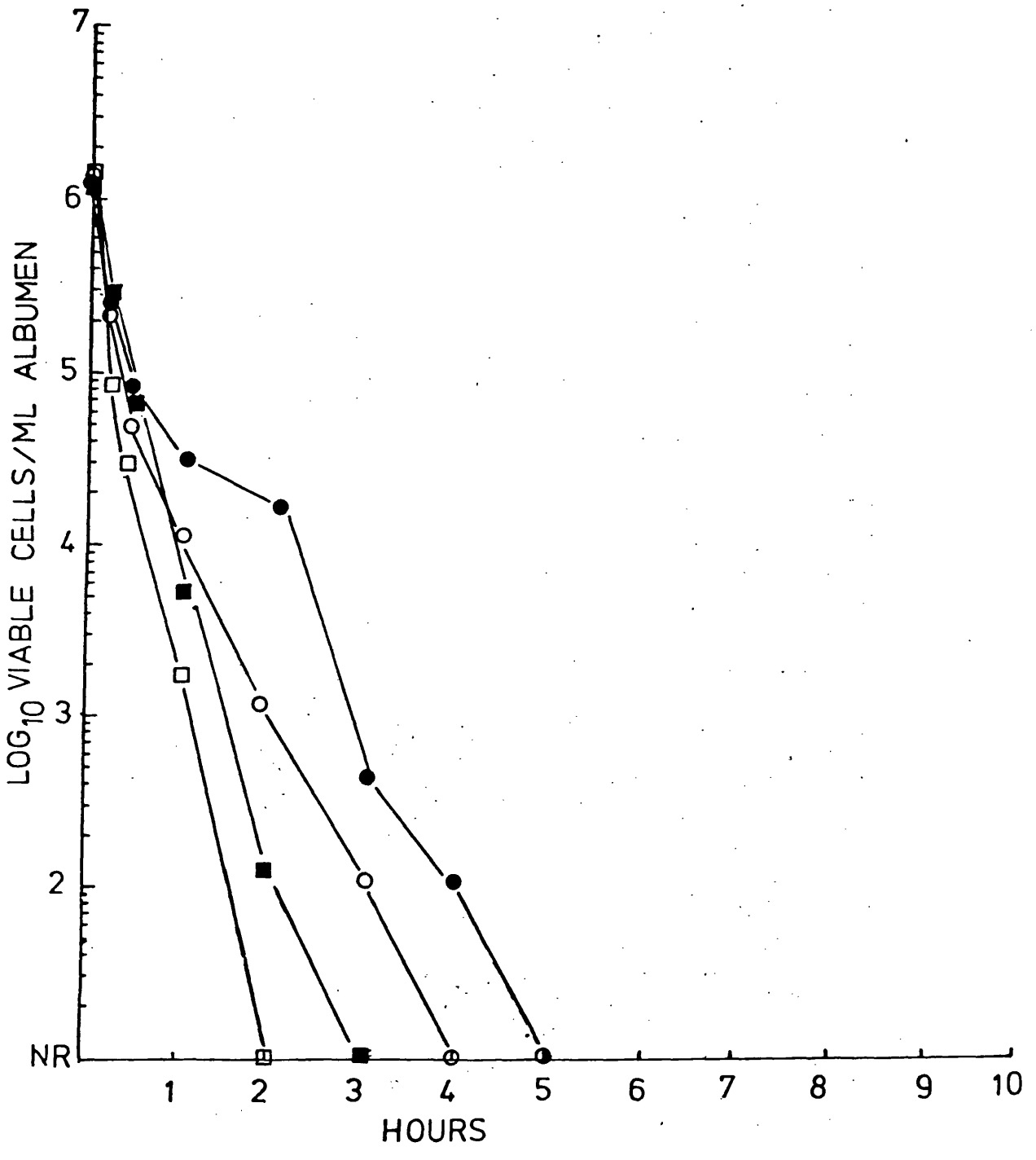


Fig. 25. The fate of Bacillus megaterium in hen egg white (open symbols) and iron saturated egg white (closed symbols) at 30°C (o, •) and 39.5°C (□ , ■).

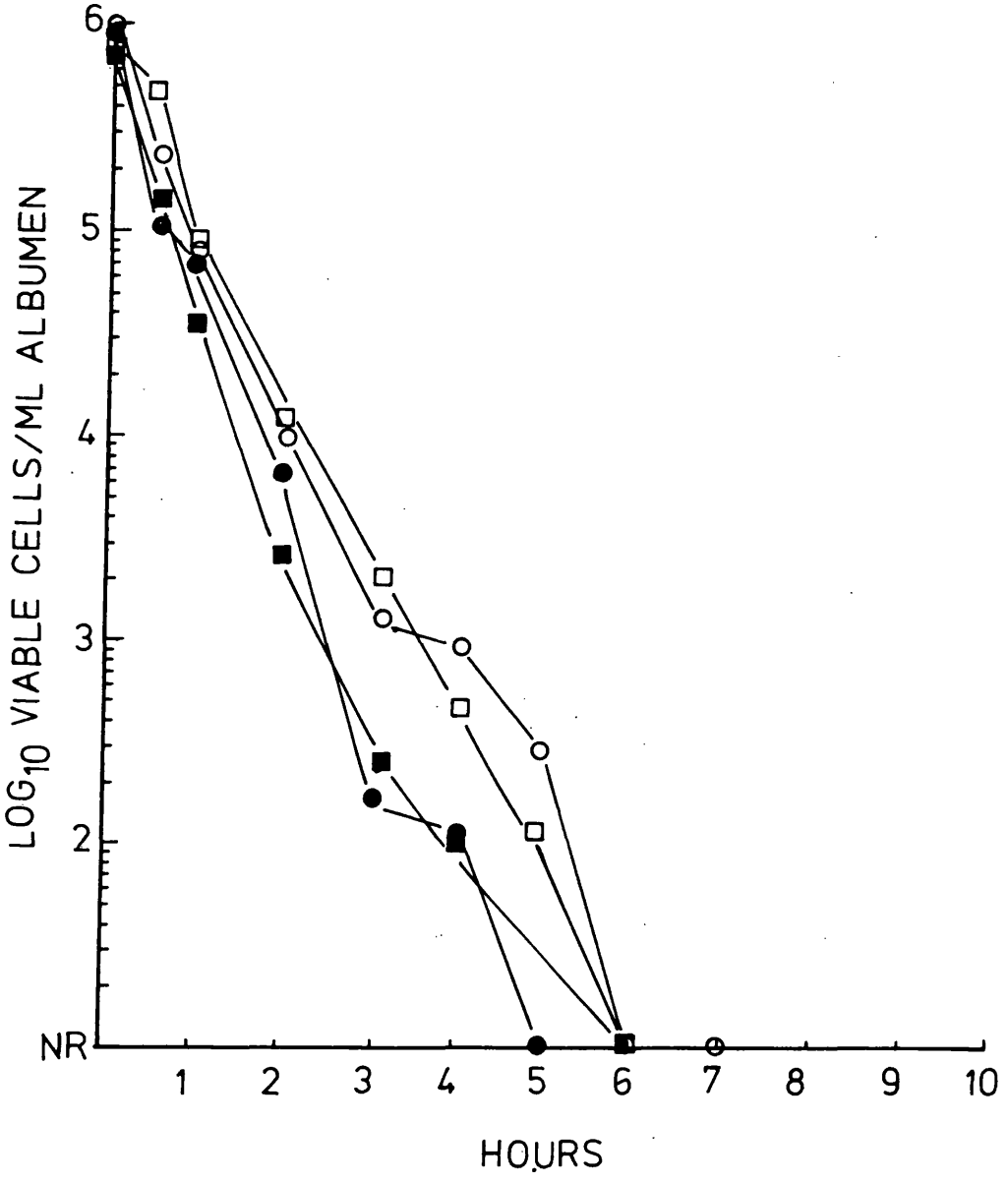


Fig. 26. The fate of Micrococcus luteus in hen egg white (open symbols) and iron saturated hen egg white (closed symbols) at 30°C (o, •) and 39.5°C (□ , ■).

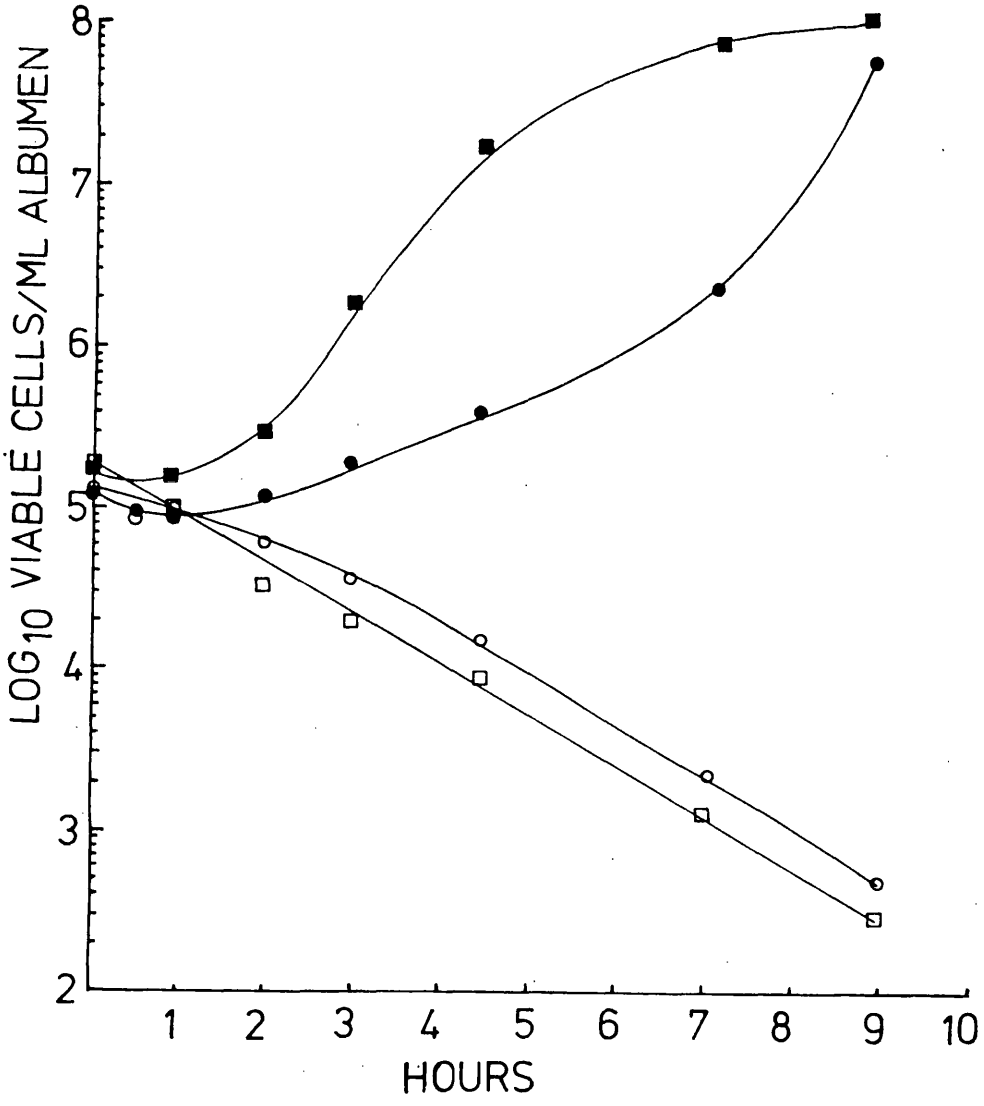


Fig. 27. The effect of iron saturation (closed symbols) on the fate of Escherichia coli 0141 (o, •) and Escherichia coli 0111 (□ , ■) in hen egg white at 39.5°C.

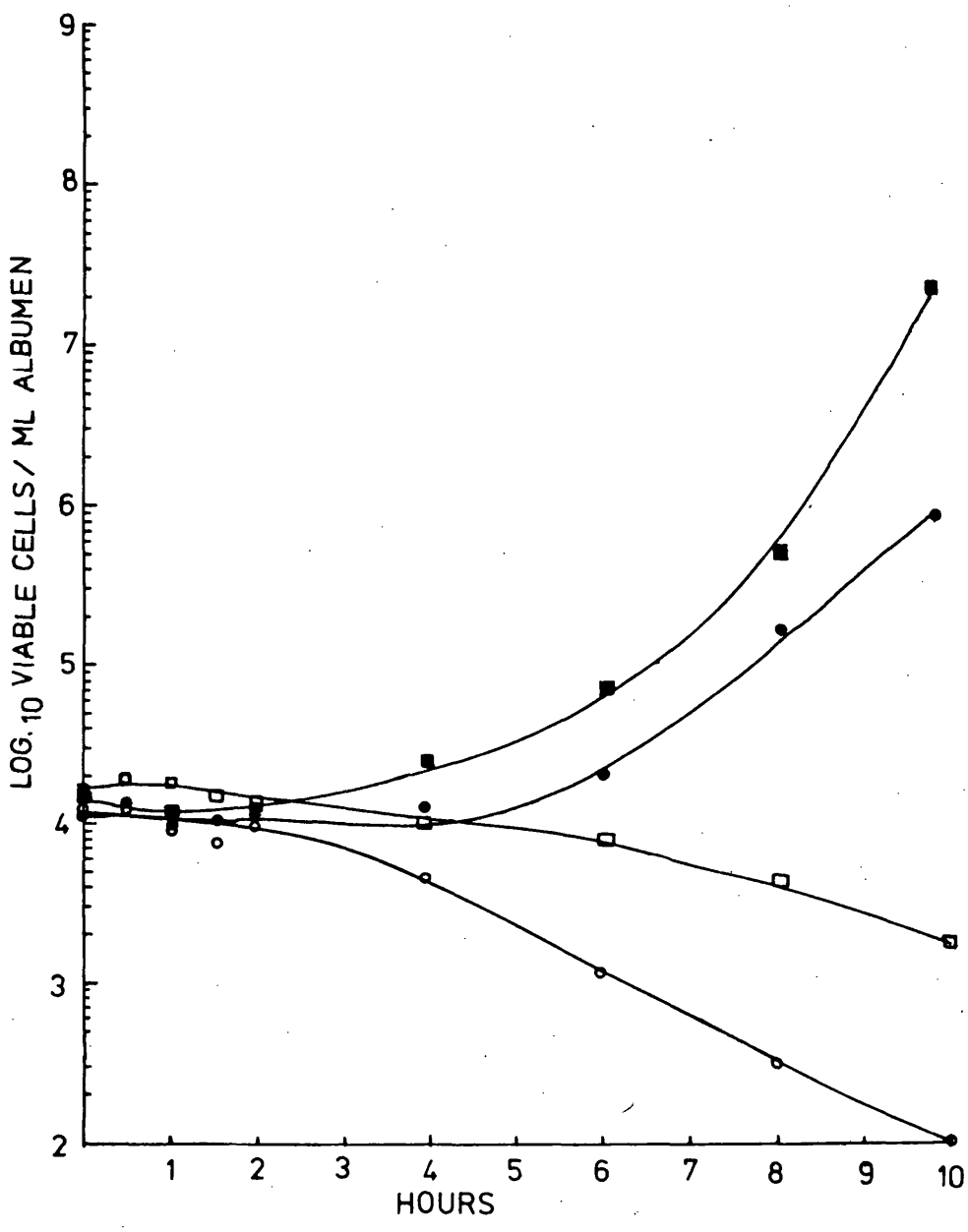


Fig. 28. The effect of iron saturation (closed symbols) on the fate of Escherichia coli C20 (o, •) and Escherichia coli C3650 (□, ■) in hen egg white at 39.5°C.

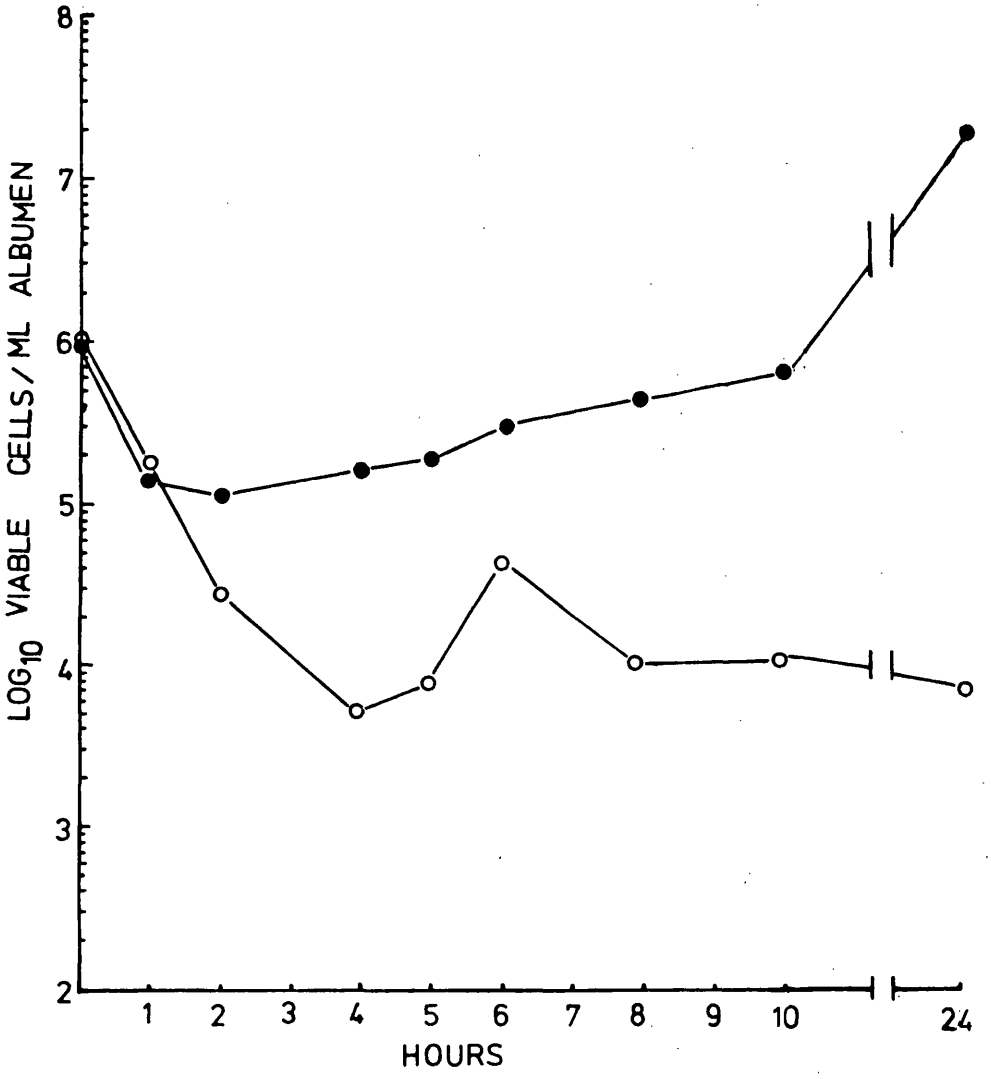


Fig. 29. The effect of iron saturation (closed symbols) on the fate of Pseudomonas aeruginosa in hen egg white at 39.5°C.

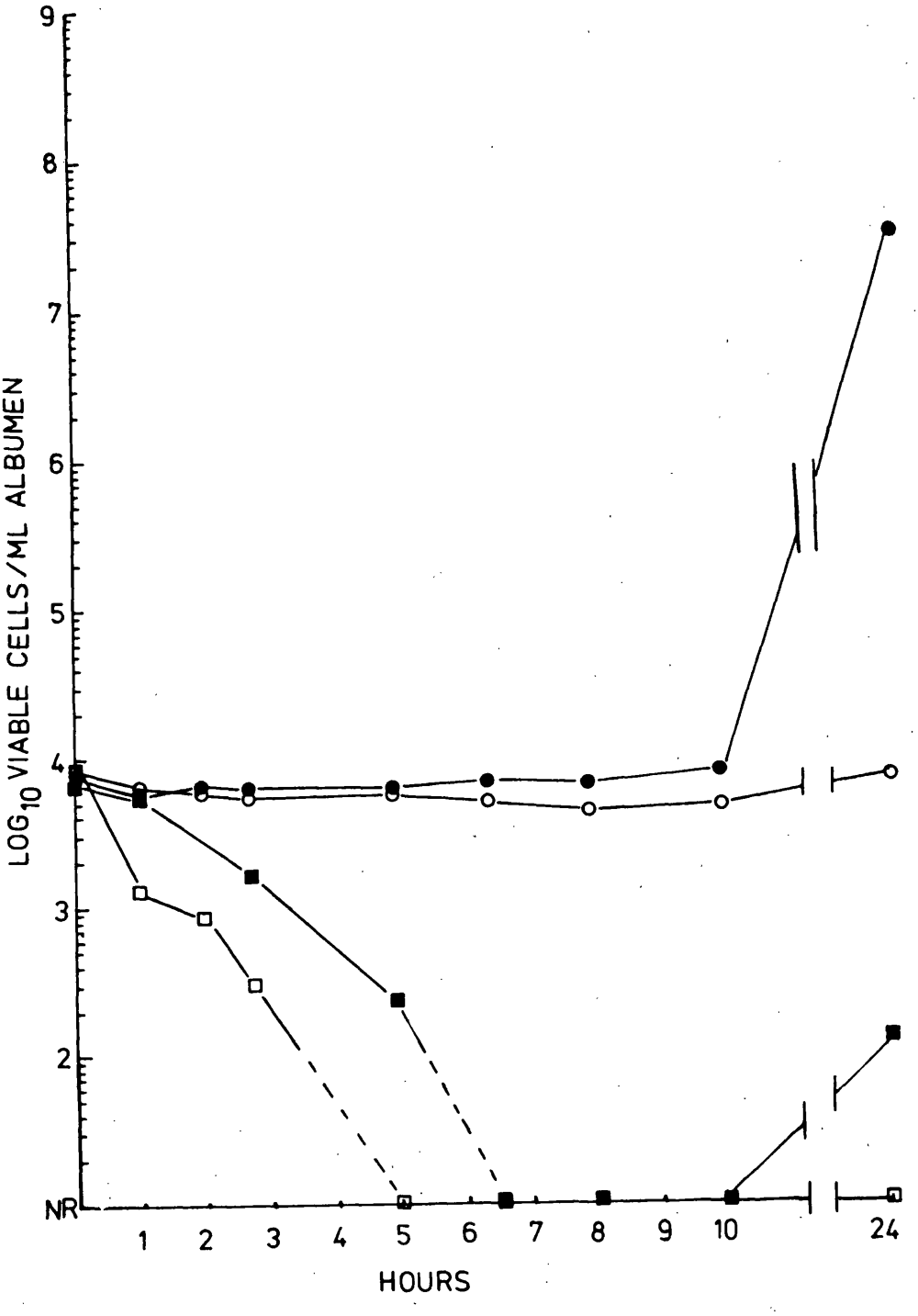


Fig. 30. The effect of iron saturation (closed symbols) on the fate of Enterobacter aerogenes in hen egg white at 30°C (o, •) and 39.5°C (□, ■).

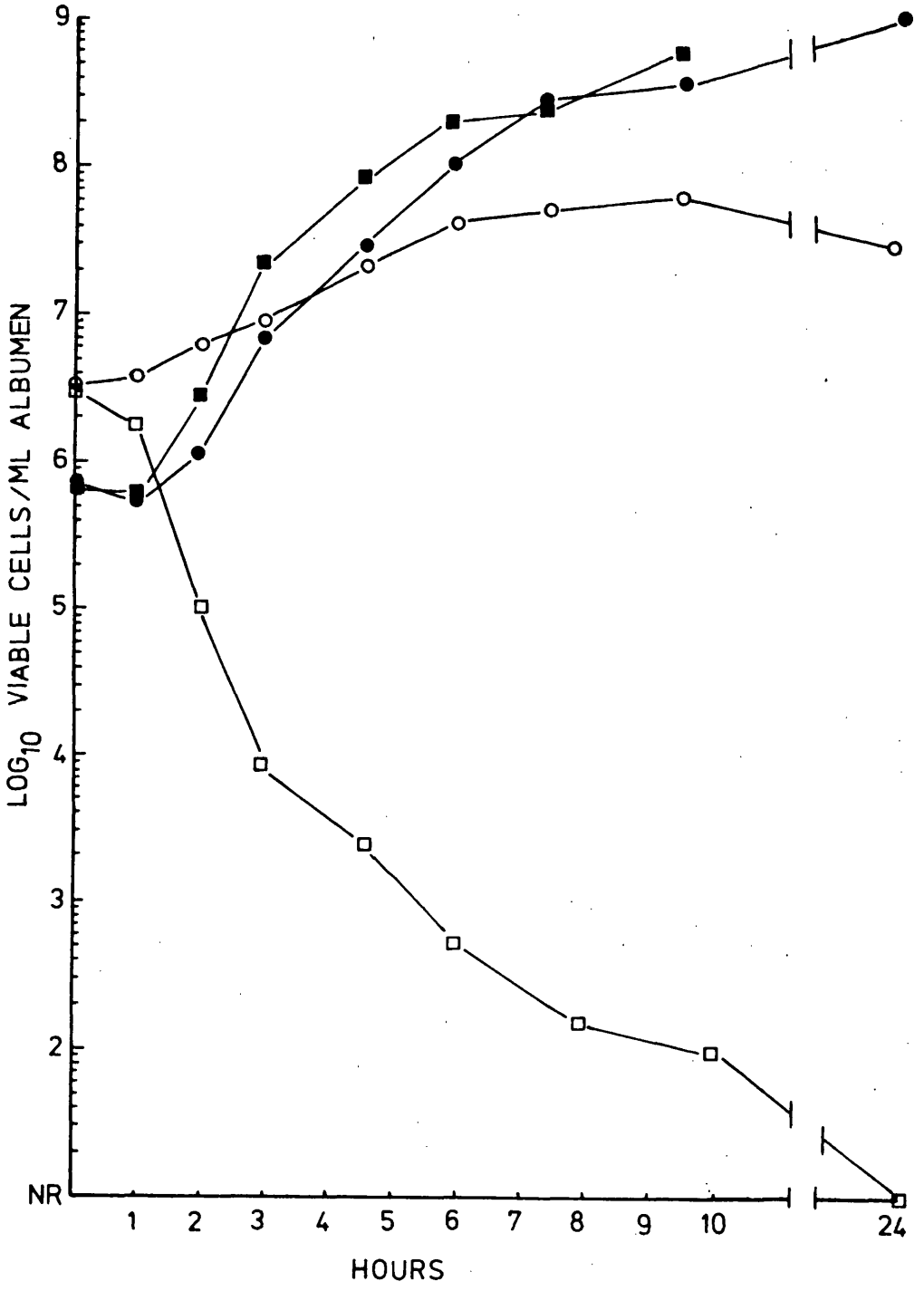


Fig. 31. The effect of iron saturation (closed symbols) on the fate of Salmonella dublin in hen egg white at 30°C (o, •) and 39.5°C (□, ■).

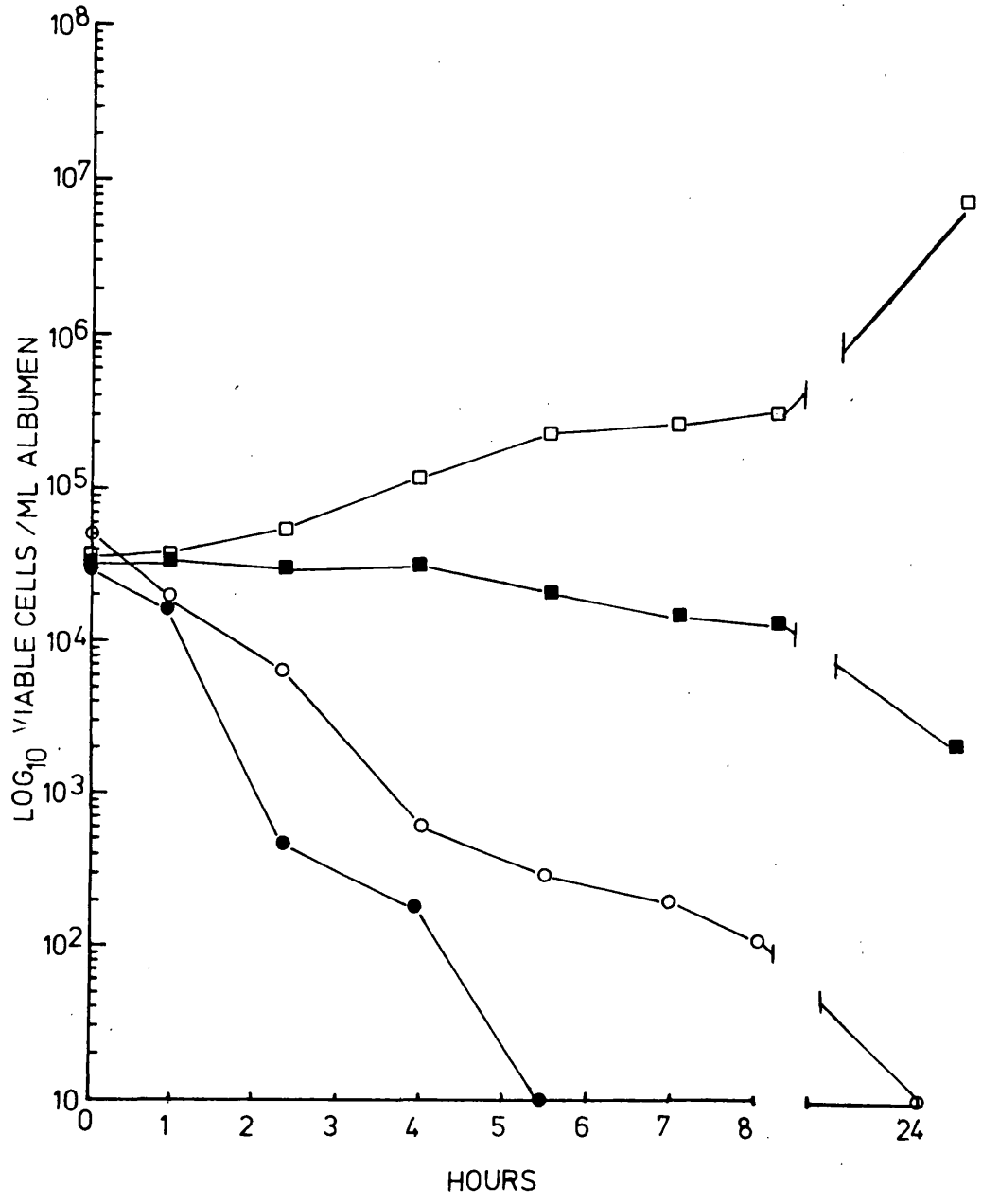


Fig. 32. The effect of temperature on the fate of Pseudomonas fluorescens in egg albumen. Pseudomonas fluorescens isolated from hatchery (open symbols) Pseudomonas fluorescens from departmental stock (closed symbols); 30°C (o, •) 39.5°C (□ , ■).

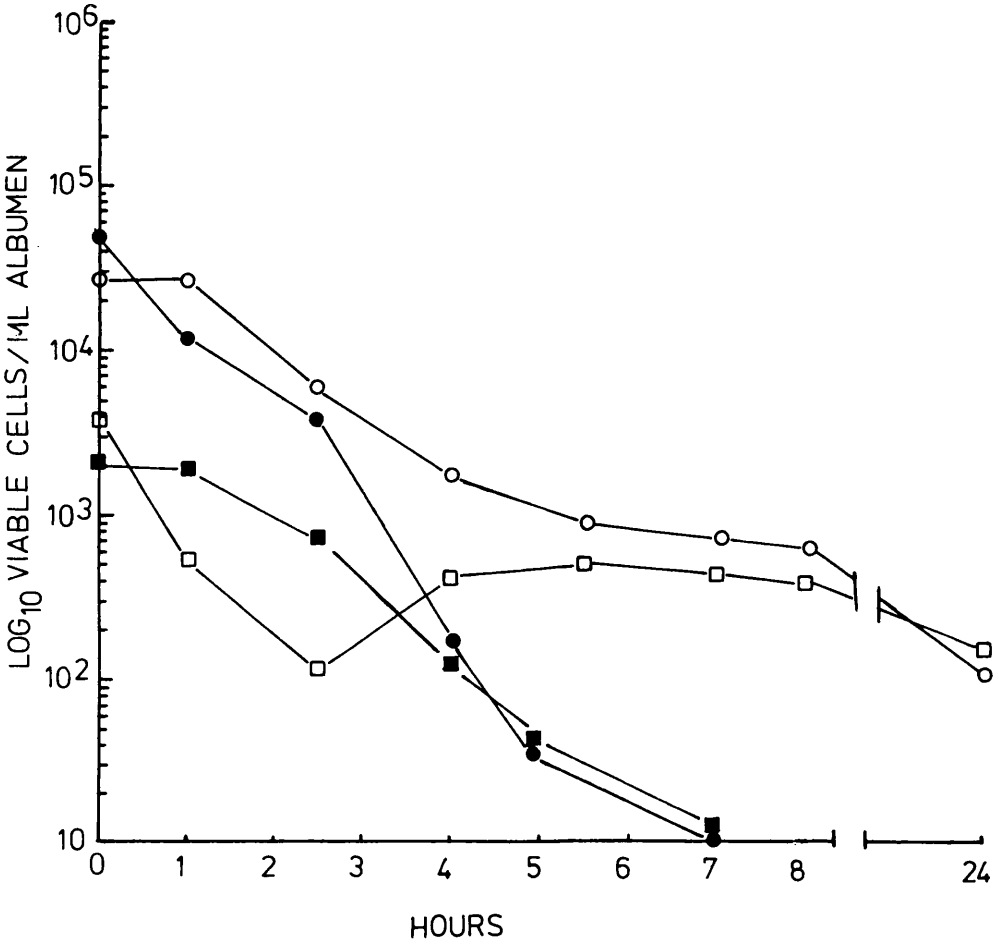


Fig. 33. The effect of temperature on the fate of Bacillus cereus in hen egg albumen. Bacillus cereus isolated from hatchery (open symbols), Bacillus cereus T (closed symbols); 30°C (o, •) 39.5°C (□ , ■).

TABLE 11

THE EFFECT OF IRON SATURATION OF OVOTRANSFERRIN ON THE FATE OF *ESCHERICHIA COLI* C3650 IN EGG WHITE AT 39.5°C

% saturation of ovotransferrin with iron *	Viable count cells /ml albumen 0h	6h	12h	24h
0	7.73×10^3	9.83×10^2	1.0×10^2	-
25	7.70×10^3	1.04×10^3	2.5×10^2	-
50	8.1×10^3	1.72×10^3	2.75×10^2	-
75	7.79×10^3	1.48×10^3	5.0×10^2	1.0×10^2
100	7.95×10^3	1.22×10^4	1.5×10^6	7.2×10^8

* The different saturation values were calculated from the amount of iron needed to give 100% saturation of hen ovotransferrin, as determined by a standard curve.

The fate of bacterial endospores in hen egg white

Apart from the paper by Laschtschenko (1909), little attention has been given to the fate of bacterial endospores in egg white. The major observations made in such a study are given in the publication bound in the back of this thesis. The following section merely notes the major features discussed in the paper and presents additional evidence, particularly that obtained from electron and light microscopy study.

(i) Germination

Stages in the development of B. cereus T spores in hen egg white and tryptone soya broth at pH 9.0 are shown in plate 6 (a-h). There was no demonstrable differences in the rate of germination (Fig. 34) of B. cereus T spores (phase bright : phase dark ; see plates a b c d) in tryptone soya broth (pH 9.0) and hen egg white (pH 9.0) supplemented with L-alanine and inosine.

(ii) Swelling

It was noted (Tranter and Board, 1982) that the volume of B. cereus T spores increased to a greater extent in hen egg white (pH 9.0) than in tryptone soya broth (pH 9.0). The swelling of these spores occurred in two distinct phases (see Fig. 2 , Tranter and Board, 1982). In the first phase the increase in volume resulted from an increase in both the length and breadth of the spores in egg white and tryptone soya broth. In the second phase the spores in egg white continued to increase for about 4h both in length and breadth resulting in a swollen "balloon-like" form (plate 6f). Those in tryptone soya broth increased only in length. Thus the spore had an elongated appearance just before vegetative cell emergence began at 70 - 80 min (plate 6e).

Electron microscopy of B. cereus T spores in egg white and tryptone

soya broth did not show any major differences in morphology although in some cases the core in spores obtained from egg white had expanded to such an extent that the cortex had been squeezed right against the spore coats and was almost indistinguishable from the latter (plates 7ab and 8ab).

(iii) Vegetative cell outgrowth

Vegetative cells of B. cereus T emerged normally from spores inoculated into tryptone soya broth at pH 7.6 and 9.0 (plate 9a and c). No free vegetative cells emerged from spores suspended in egg white pH 7.6 and 9.0 within three hours of inoculation. A small percentage (approx. 2%) of the spores attempted outgrowth but the cells were always associated with the spore coat and appeared structurally very weak (plate 9d). When left overnight, some of the spores in egg white (pH 7.6) formed free cells (plate 9b) but these differed from those in tryptone soya broth in size and shape and in many cases cell division appeared to be affected (plate 9e). Although the vegetative cells in egg white were of a bizarre morphology they gave a Gram-positive stain reaction (plate 10) and moreover they contained a small amount of lipid granules (plate 11).

As with E. coli C20 (Fig. 20) the addition of casamino acids, growth factors or trace elements to egg white did not effect the events depicted in plate 6 (e-h). At pH 7.6 however iron removed the inhibition of outgrowth (plate 12). Indeed cells formed in iron-supplemented egg white had a similar morphology to those formed from spores in a rich medium such as tryptone soya broth. Although the cells in egg white stained Gram-positive, the staining was very granular (plate 12) due to the presence of large amounts of lipid present in the cytoplasm (plate 13). When left overnight many of the cells formed spores

(plate 14). Iron had no effect on the inhibition of outgrowth by egg white at pH 9.0.

Electron microscopy of the outgrowth process from spores in tryptone soya broth (pH 9.0) revealed normal emergence of the free cells (plate 15). The cells emerging from spores in egg white (pH 9.0) appeared distorted and in many cases lysis of the cell envelope seemed to be occurring as the cells emerged from the spore coat. In some of the preparations the membrane of emerging vegetative cells from spores in egg white appeared to be disrupted and incapable of maintaining cell integrity (plate 16a and b).

These observations together with the evidence presented in the publication bound in the back of this thesis suggest that the inhibition of outgrowth from spores of B. cereus T suspended in hen egg white is dependent upon both the high pH of the medium and the absence of a freely available source of iron. Similar results obtained from spores suspended in a simple defined medium of a high pH in the presence of pure hen ovotransferrin support these conclusions.

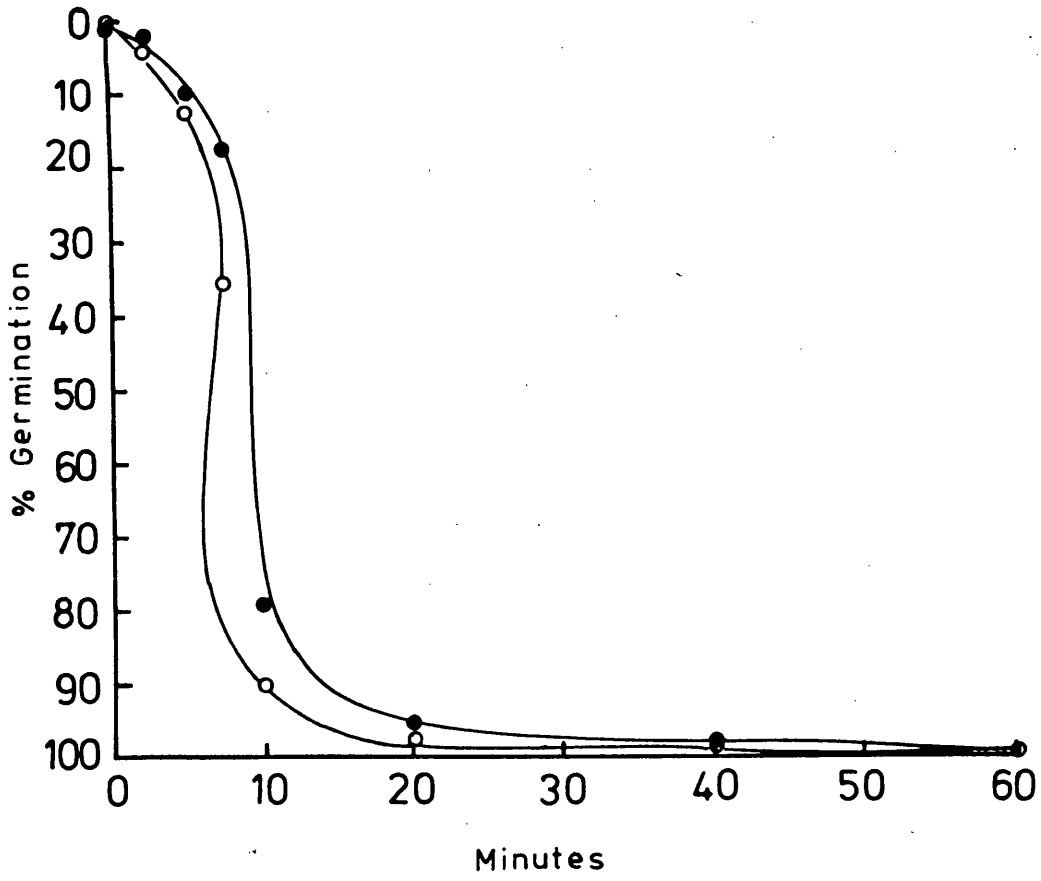
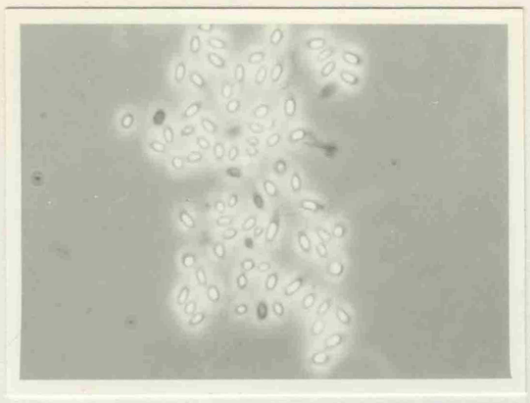
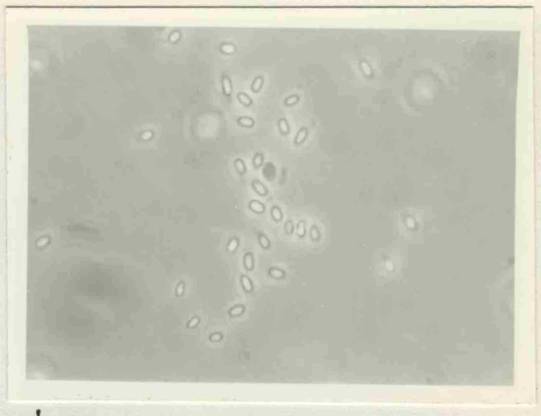


Fig. 34. Germination of Bacillus cereus T spores in tryptone soya broth (●) and hen egg white (o) at pH9.0.

Plate 6a-h. Phase contrast micrographs of stages in the development of Bacillus cereus T spores in tryptone soya broth (a,c,e,g) and hen egg white (b,d,f,h) at pH9.0; (a and b) mostly ungerminated spores ; (c and d) mostly germinated spores ; (e and f) swelling before outgrowth stage ; (g) outgrowth of vegetative cells in tryptone soya broth ; (h) second swelling stage of spores in egg white.



a



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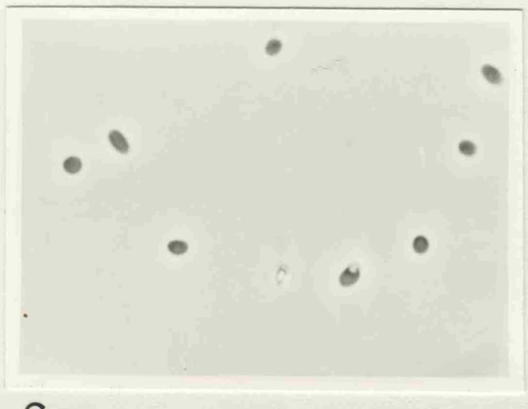

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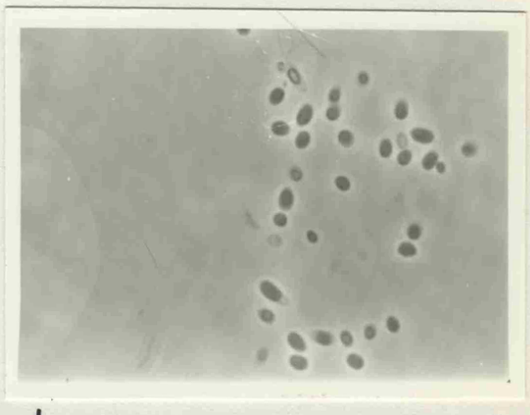

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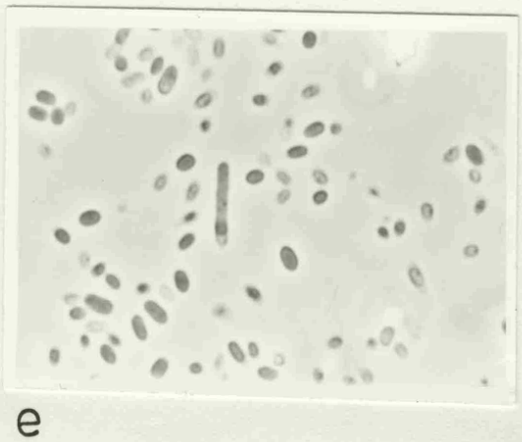
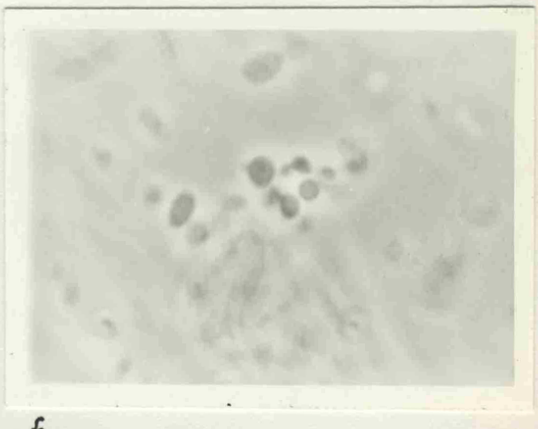
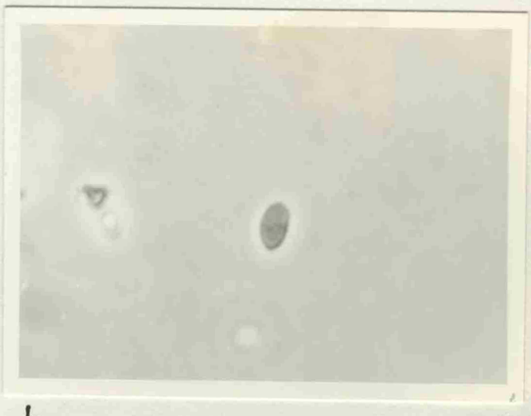


e



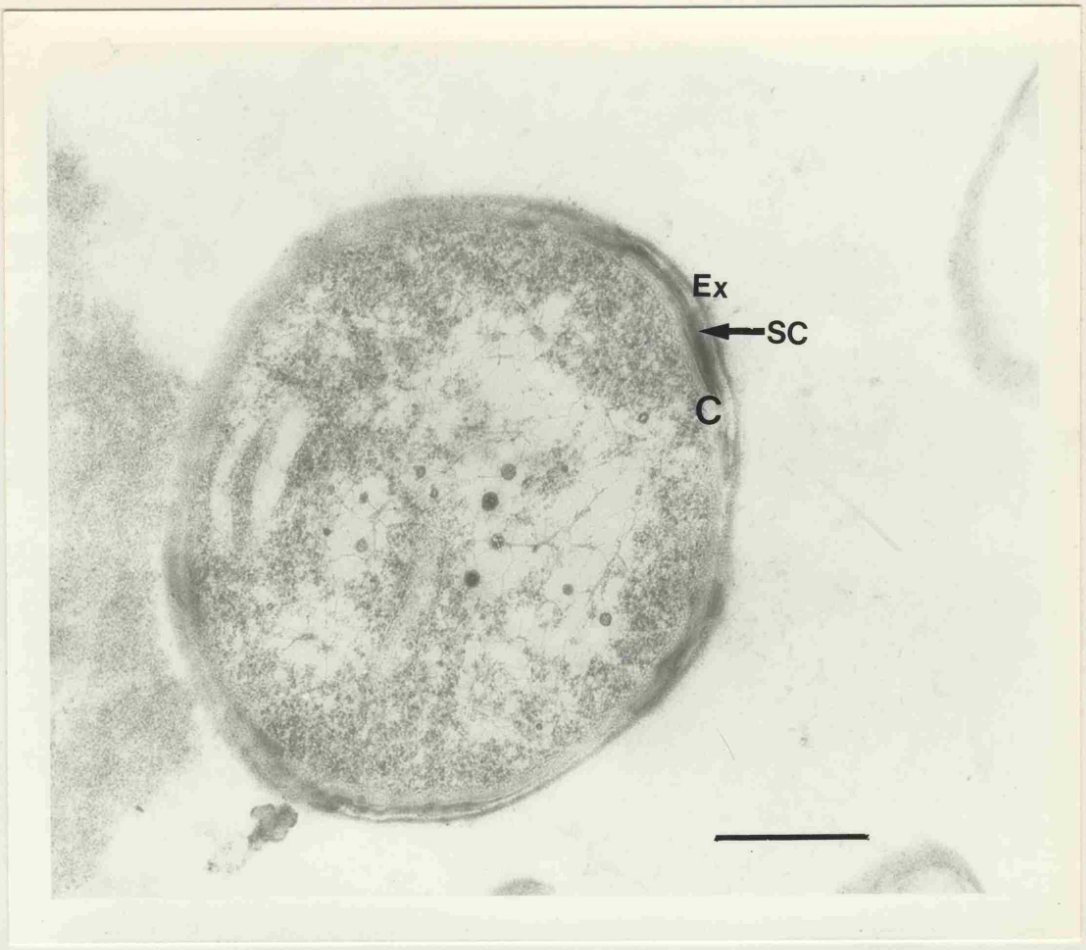
f

g



h

Plate 7a and b. Electron micrographs of Bacillus cereus T spore in hen egg white showing swelling of spore cortex (C) within the spore coats (SC) and exosporium (Ex). In b the cortex is indistinguishable from the spore coats. The bar marker represents 1 um.



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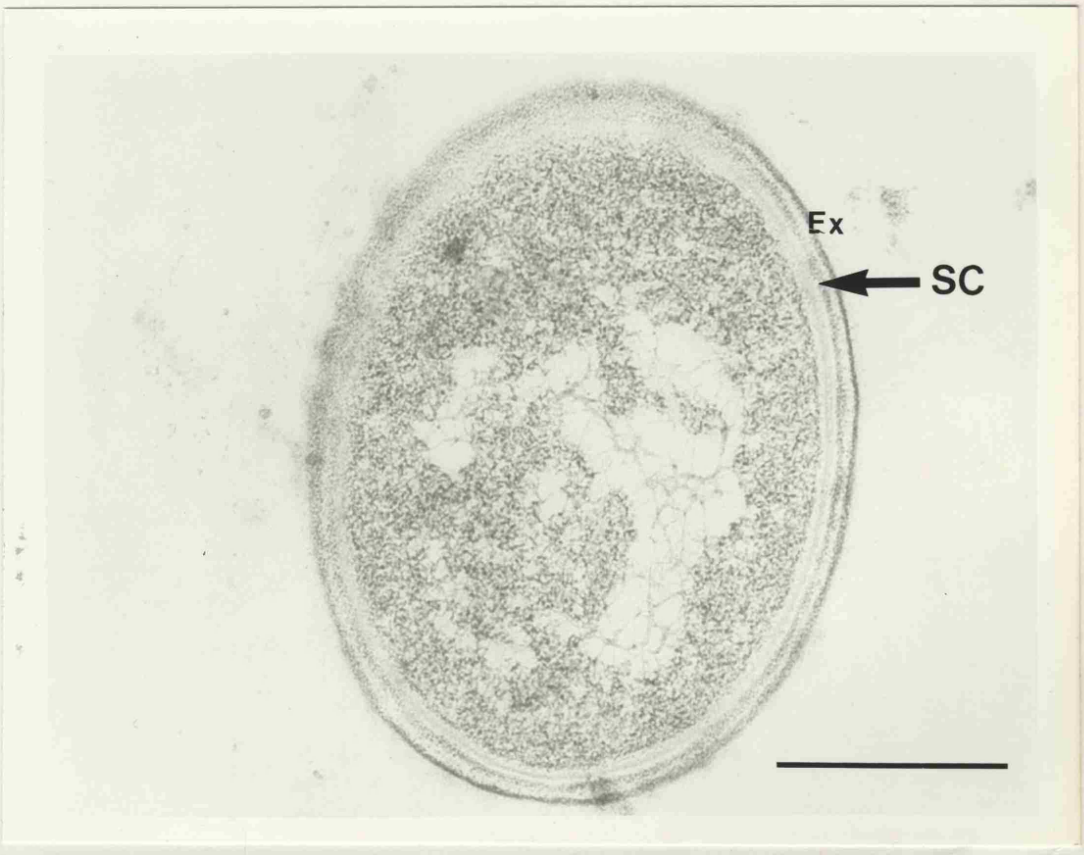
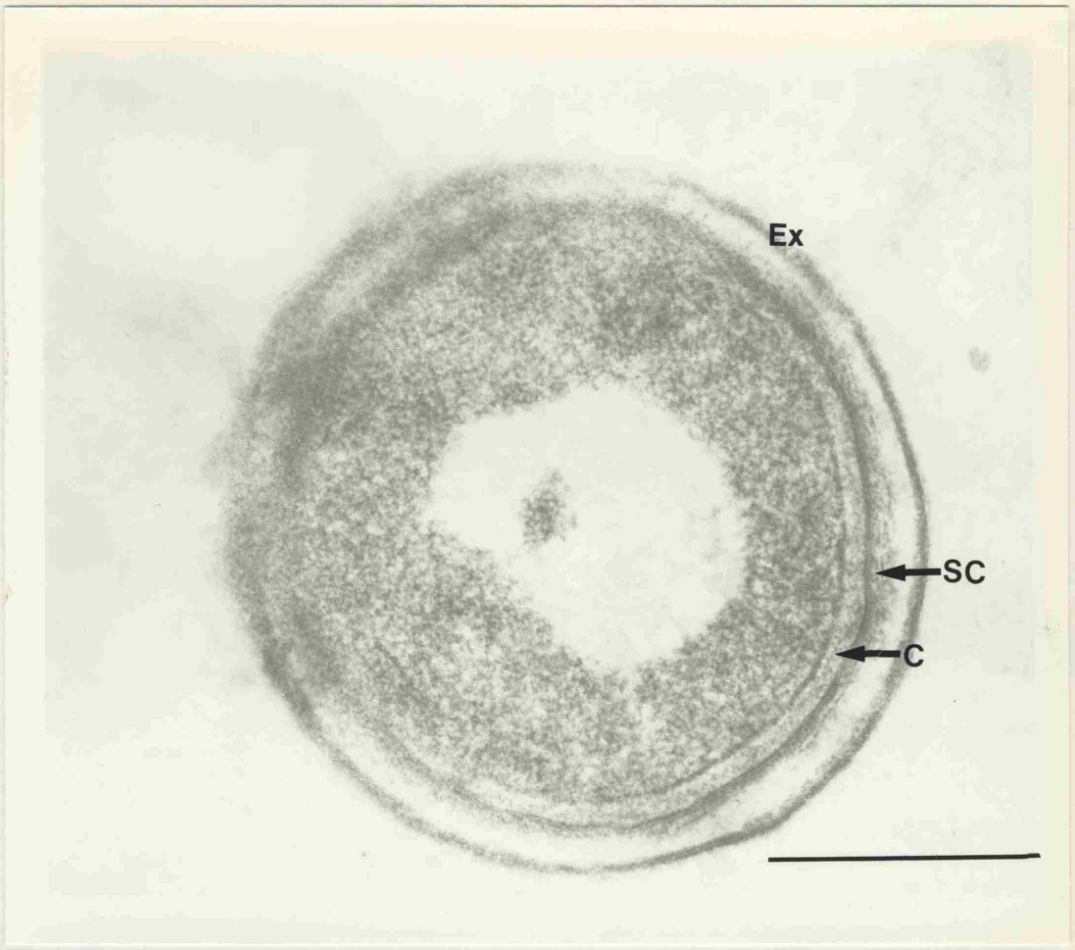


b

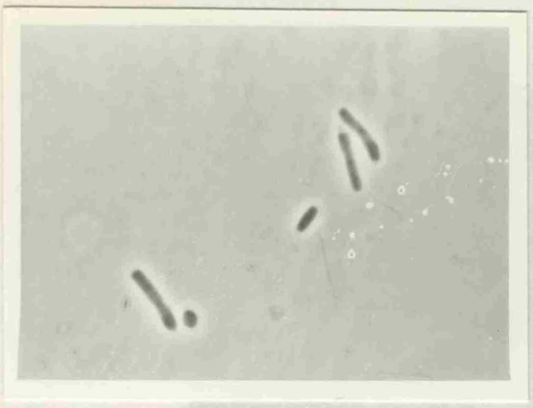
Plate 8a and b. Electron micrographs of Bacillus cereus T spores in tryptone soya broth pH9.0. Although swelling has occurred the cortex (C) is still visible within the spore coats (SC) and exosporium (Ex). The bar markers represent 1 μ



a

b

Plate 9a-e. Outgrowth of vegetative cells of Bacillus cereus T from spores suspended in tryptone soya broth (a and c) and hen egg white (b and d) at pH9.0 (c and d) and 7.6 (a and b). In many cases cell division appeared to be affected during outgrowth in egg white pH7.6 (e).



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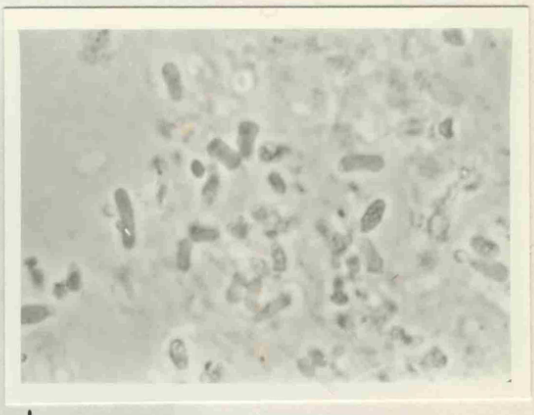

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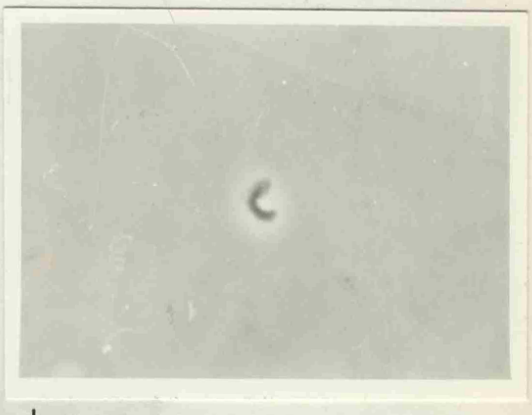


d

e

Plate 10. Gram stain of vegetative cells of Bacillus cereus T that have grown out of spores suspended in hen egg white pH7.6 after 18h.

Plate 11. Vegetative cells of Bacillus cereus T that have grown out of spores suspended in hen egg white pH7.6 after 18h, stained with Burdon's lipid stain to demonstrate the presence of lipid granules.

Plate 12. Gram stain of vegetative cells of Bacillus cereus T that have grown out of spores suspended in hen egg white for 18h at pH7.6 saturated with iron.

Plate 13. Vegetative cells of Bacillus cereus T that have grown out of spores suspended in hen egg white for 18h at pH7.6 supplemented with iron stained with Burdon's lipid stain to show the presence of granules.

Plate 14. Phase contrast micrograph of vegetative cells of Bacillus cereus T that have grown out of spores suspended in hen egg white for 18h at pH7.6 saturated with iron showing the presence of light refractive spores.

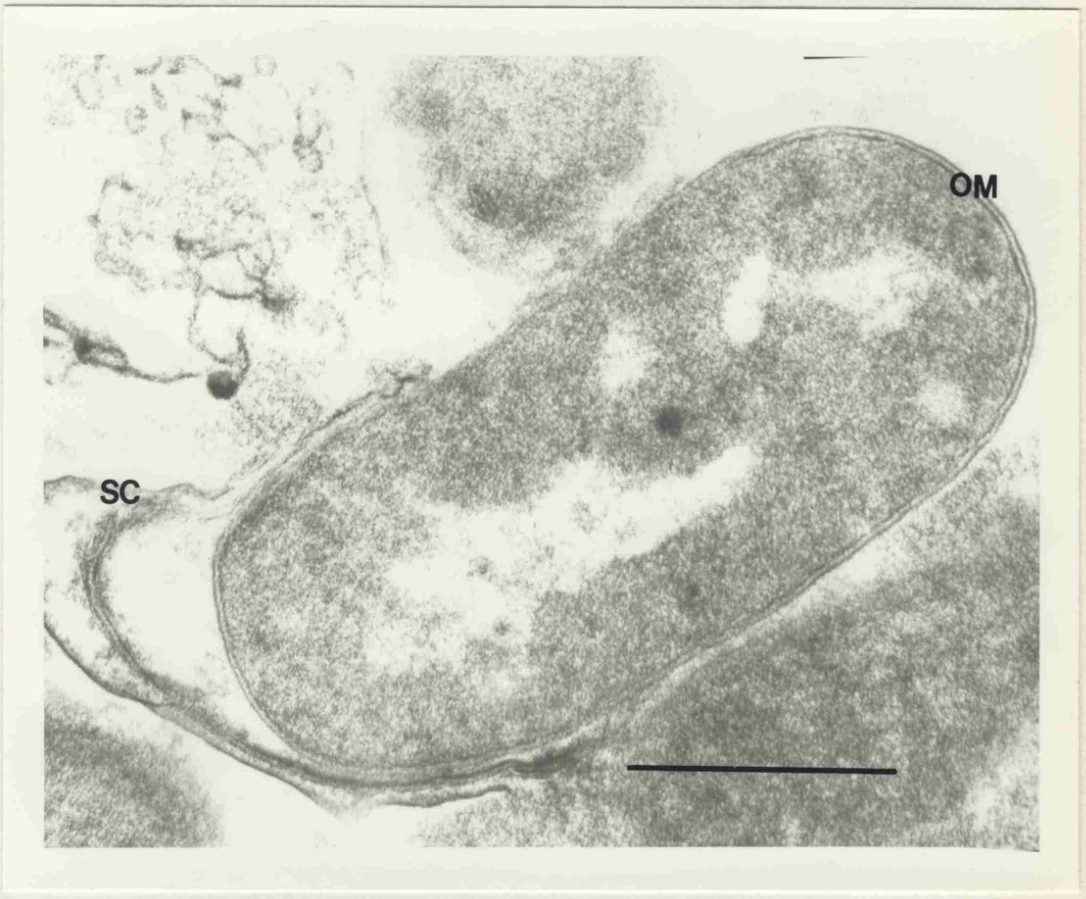
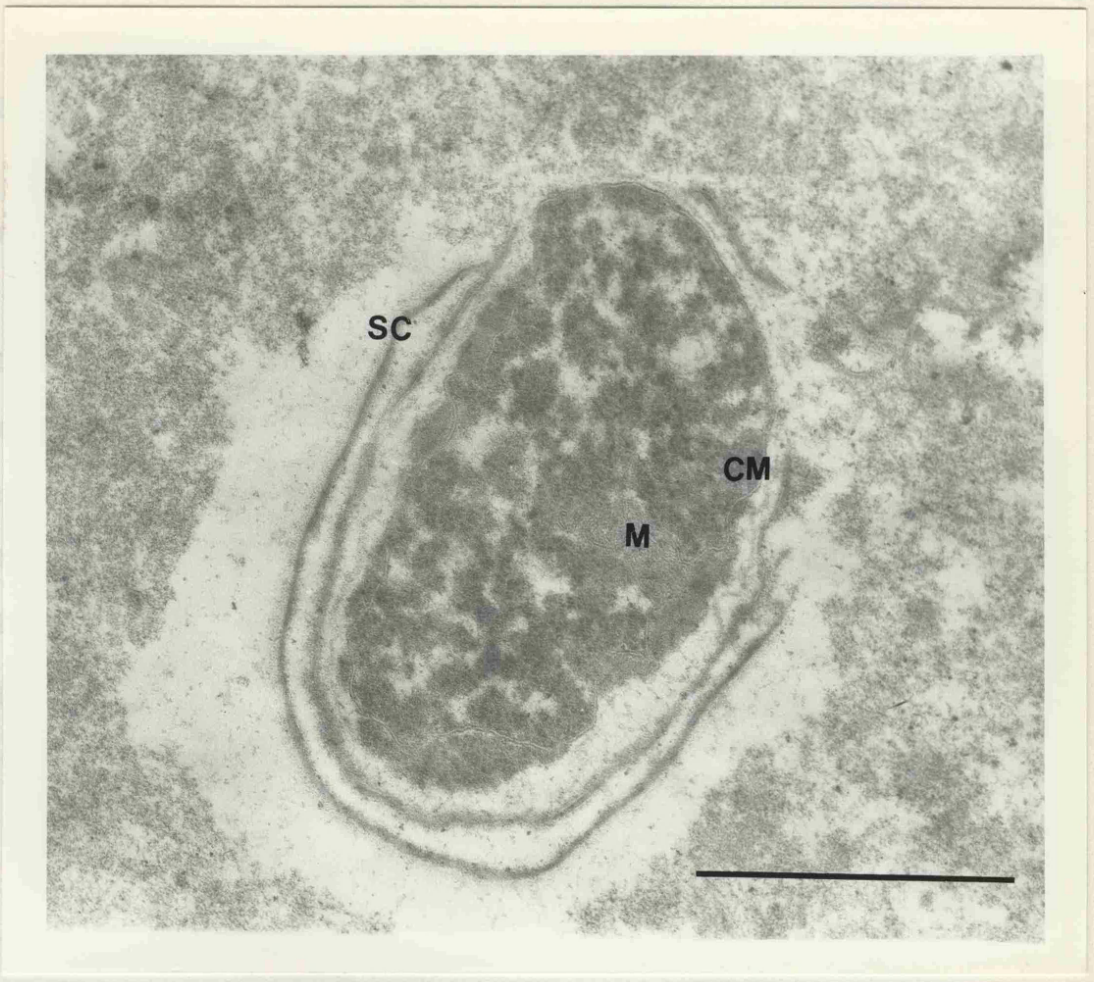


Plate 15. Electron micrograph showing the normal emergence of a vegetative cell from the spore coat (SC) of Bacillus cereus T spores suspended in tryptone soya broth pH9.0. The outer membrane (OM) appears intact and unaffected. The bar marker represents 1μ

Plate 16a and b. The emergence of vegetative cells of Bacillus cereus T from spores suspended in hen egg white pH9.0. The cells appear to have distorted cell membranes (CM) and although mesosomes (M) are clearly visible most of the cells are unable to leave the spore coat (SC). The bar markers represent 1μ



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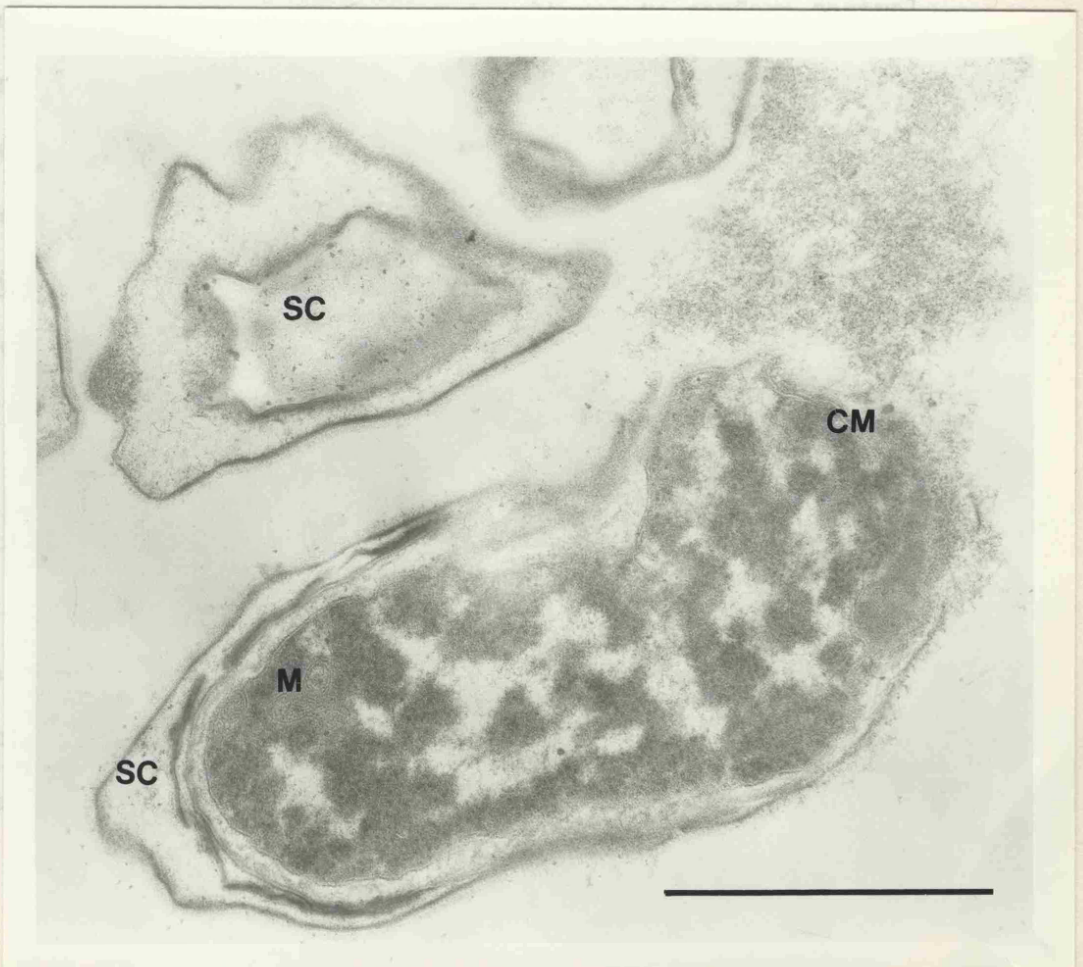

b

The fate of yeast vegetative cells in hen egg white

In most cultures of yeast cells the occurrence of clumps of cells presents a problem which complicates the making and interpretation of total cell counts, viable counts and relative numbers of budded and unbudded cells (see Pringle and Mor, 1975). The degree of clumping varies greatly both with the strain and culture conditions. In most cultures one not only sees budded cells (a forms) and mother plus daughter cells (b forms) but clumps of various sizes ranging from c-f (Fig. 35). In each b form the mother and daughter are joined through a continuous cytoplasm and the two forms can not be pulled apart. In contrast all c and d forms can be easily separated into a and b forms without damaging the cells in any detectable way or reducing their viability. This separation can be achieved particularly well by sonication, the amount of which varies depending upon the strain, power setting, tuning of the instrument etc. It is necessary before doing viable counts on yeast cultures to perform control experiments, such as those in Tables 12-16, in the medium to be used to determine how much sonication is sufficient in a given situation. While it can be seen that some strains e.g. Debaryomyces hansenii and Candida krusei still contain clumps even after prolonged sonication in both Y.E.P.G. medium and egg white, 60 s of sonication was generally sufficient to eliminate high amounts of clumping in both media.

The effect of hen egg albumen on the growth of yeast vegetative cells (Figs. 36-39) was very similar to the effects observed with both bacterial vegetative cells and endospores. The incubation temperature, pH and iron availability all contributed to the general decrease in viable numbers. However, with the exception of Saccharomyces cerevisiae in albumen (pH 7.5) (Fig. 38), iron saturation of the white's content

of ovotransferrin did not lead to growth of the yeast concerned, although in the majority of cases it did not enhance the death of the cells. In marked contrast to bacterial cells where the addition of iron overcame any inhibitory effects at temperatures and pHs within the tolerance of the bacteria concerned, the dependence upon a freely available source of iron did not seem to be the major determining factor in the case of yeast cells in hen egg white. As with bacterial vegetative cells the higher pH and incubation temperature generally increased the death of the yeast cells.

TABLE 12

THE EFFECT OF SONICATION ON CLUMPING OF CELLS OF BRETANOMYCES ANOMALUS IN YEPG MEDIUM AND HEN EGG WHITE.

	YEPG MEDIUM			HEN EGG WHITE		
SONICATION TIME (SECS.)	CLUMPS	a FORMS	b FORMS	CLUMPS	a FORMS	b FORMS
0	24	6	70	10	34	56
5	13	16	71	0	45	55
10	10	15	75		ND*	
20	7	44	49		ND	
30	2	34	64		ND	

* Not Determined

TABLE 13

THE EFFECT OF SONICATION ON CLUMPING OF CELLS OF SACCHAROMYCES CEREVISIAE IN YEPG MEDIUM AND HEN EGG WHITE.

	YEPG MEDIUM			HEN EGG WHITE		
SONICATION TIME (SECS.)	CLUMPS	a FORMS	b FORMS	CLUMPS	a FORMS	b FORMS
0	17	42	41	86	12	2
5	2	62	36	67	18	15
10	1	68	31	47	26	27
20	1	69	30	24	50	26
30	0	70	30	23	40	37
45	0	70	30	16	46	38
60	0	70	30	12	56	32

TABLE 14

THE EFFECT OF SONICATION ON CLUMPING OF CELLS OF CANDIDA VALIDA IN YEPG MEDIUM AND HEN EGG WHITE

	YEPG MEDIUM			HEN EGG WHITE		
SONICATION TIME (SECS.)	CLUMPS	a FORMS	b FORMS	CLUMPS	a FORMS	b FORMS
0	95	1	4	62	9	29
5	79	2	19	19	59	32
10	72	2	21	19	43	38
20	56	3	36	7	67	25
30	55	6	39	0	82	18
45	30	18	52	0	84	16
60	24	24	52	0	85	15

TABLE 15

THE EFFECT OF SONICATION ON CLUMPING OF CELLS OF DEBARYOMYCES HANSENII IN YEPG MEDIUM AND HEN EGG WHITE

	YEPG MEDIUM			HEN EGG WHITE		
SONICATION TIME (SECS.)	CLUMPS	a FORMS	b FORMS	CLUMPS	a FORMS	b FORMS
0	74	1	15	85	5	10
5	45	9	46	56	16	28
10	48	25	27	38	20	42
20	8	31	61	14	32	54
30	8	41	52	10	47	43
45	7	42	51	10	49	41
60	8	42	50	9	53	38

TABLE 16

THE EFFECT OF SONICATION ON CLUMPING OF CELLS OF *CANDIDA KRUSEI* IN YEPG MEDIUM AND HEN EGG WHITE.

	YEPG MEDIUM			HEN EGG WHITE		
SONICATION TIME (SECS.)	CLUMPS	a FORMS	b FORMS	CLUMPS	a FORMS	b FORMS
0	4	44	52	85	5	10
5	3	53	44	72	11	17
10	0	63	37	62	10	28
20	0	65	35	56	11	33
30		ND*		36	17	47
45		ND		40	33	27
60		ND		40	32	28
120		ND		37	34	29

* Not Determined

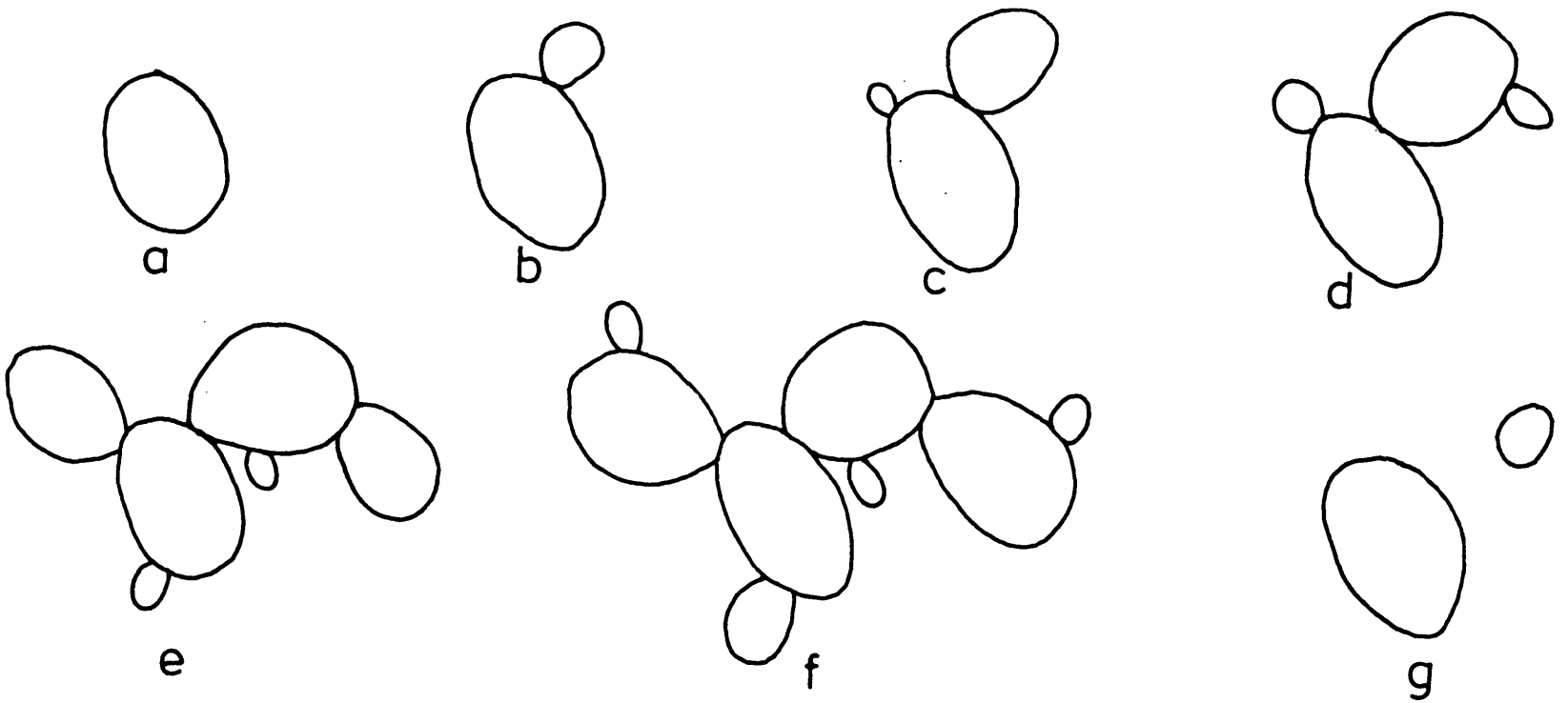

Fig. 35. Schematic representation of unbudded cells (a), budded cells and mother plus daughter pairs (b), and clumps of various sizes (c to f).

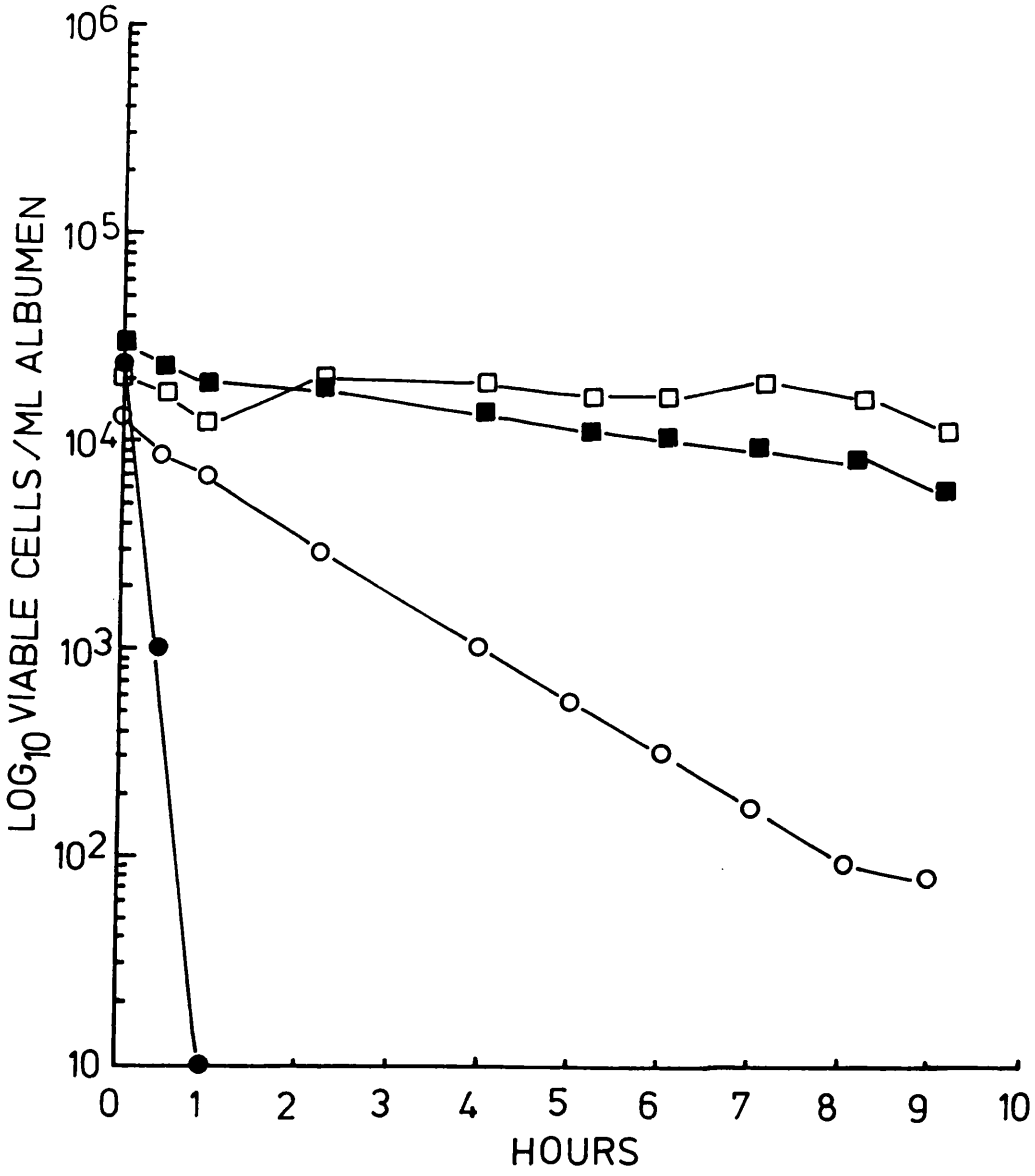

Fig. 36. The effect of incubation temperature and pH on the fate of Debaryomyces hansenii in hen egg white. 30°C (□ , ■), 39.5°C (o, •) pH9.5 (closed symbols), pH7.8 (open symbols).

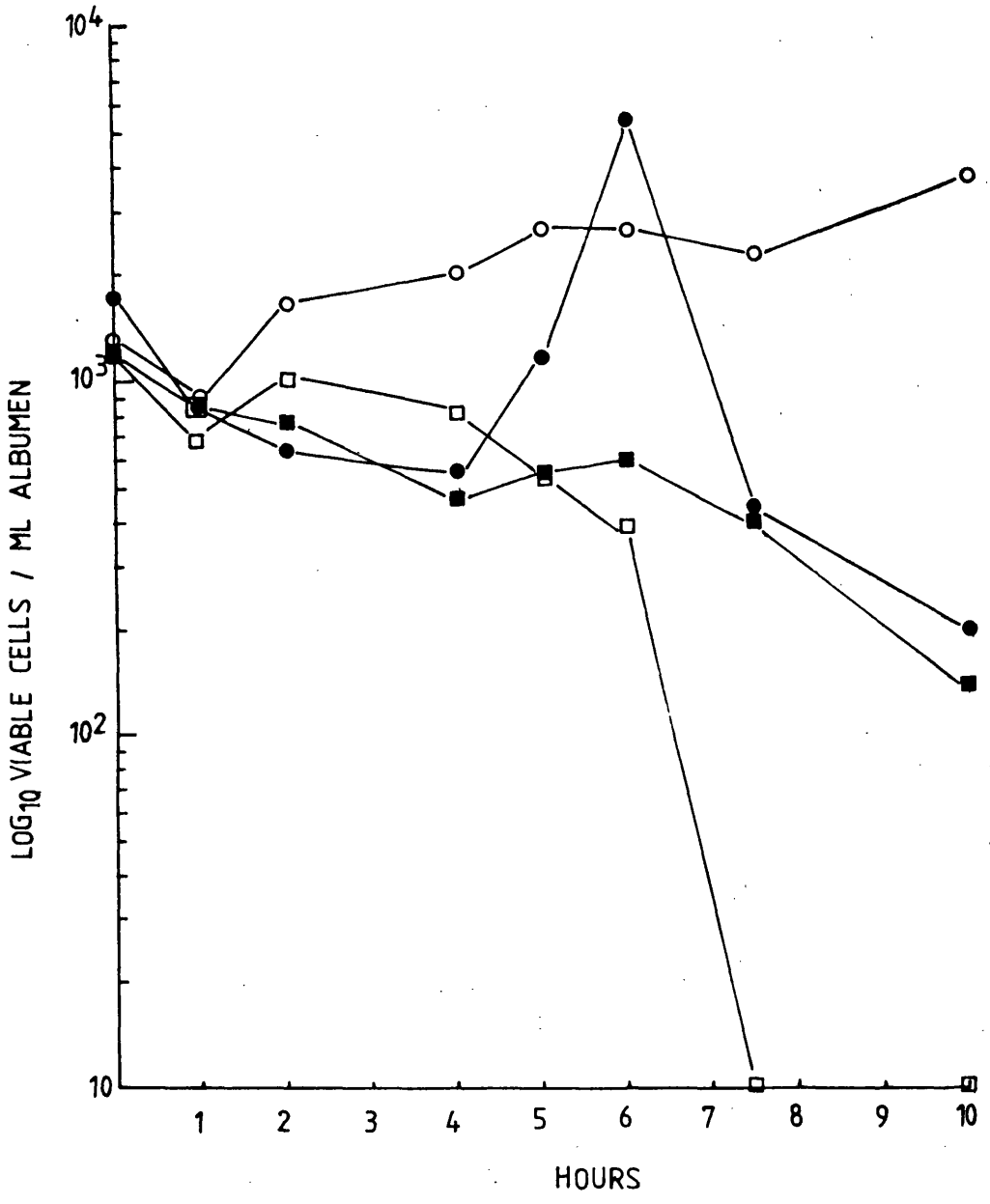

Fig. 37. The effect of incubation temperature (o, •, 30°C; □ , ■ 39.5°C) on the fate of Candida valida in hen egg white (open symbols) and hen egg white saturated with iron (closed symbols).

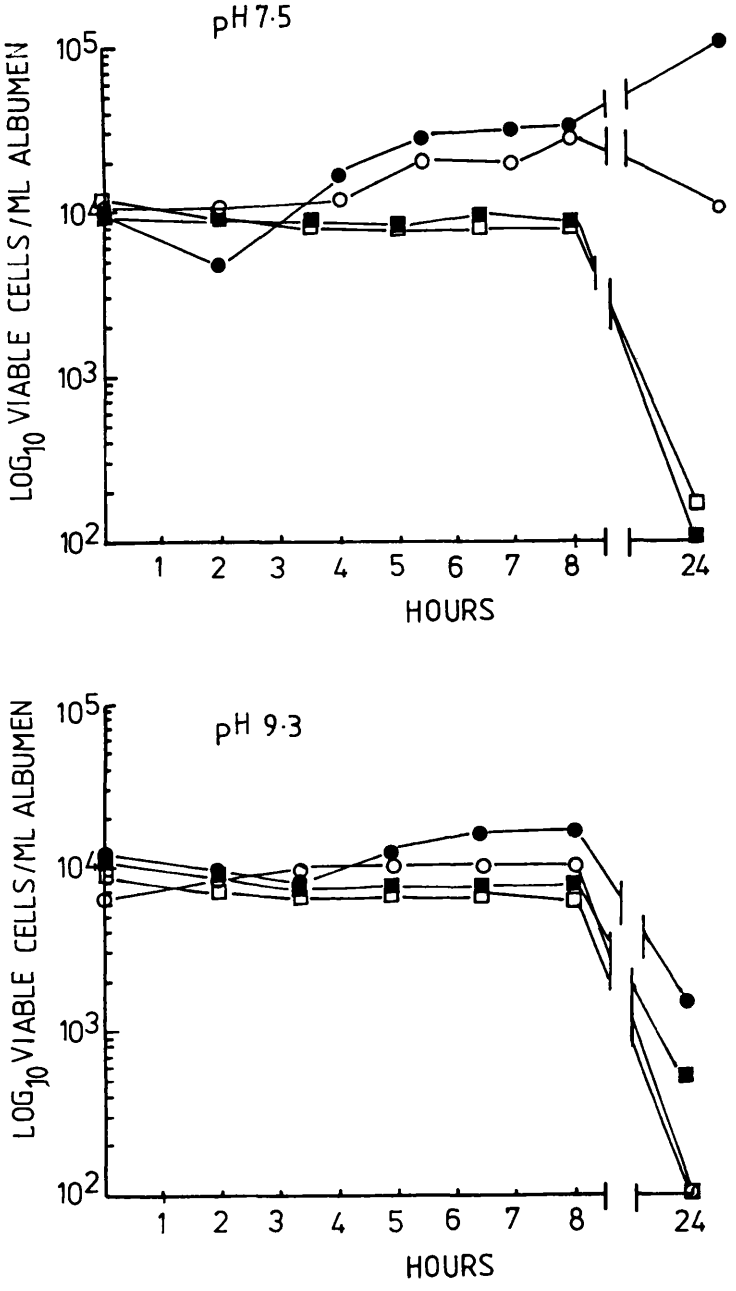

Fig. 38. The effect of incubation temperature (o, • 30°C; □ , ■ 39.5°C) and pH on the fate of Saccaromyces cerevisiae in hen egg white (open symbols) and hen egg white saturated with iron (closed symbols).

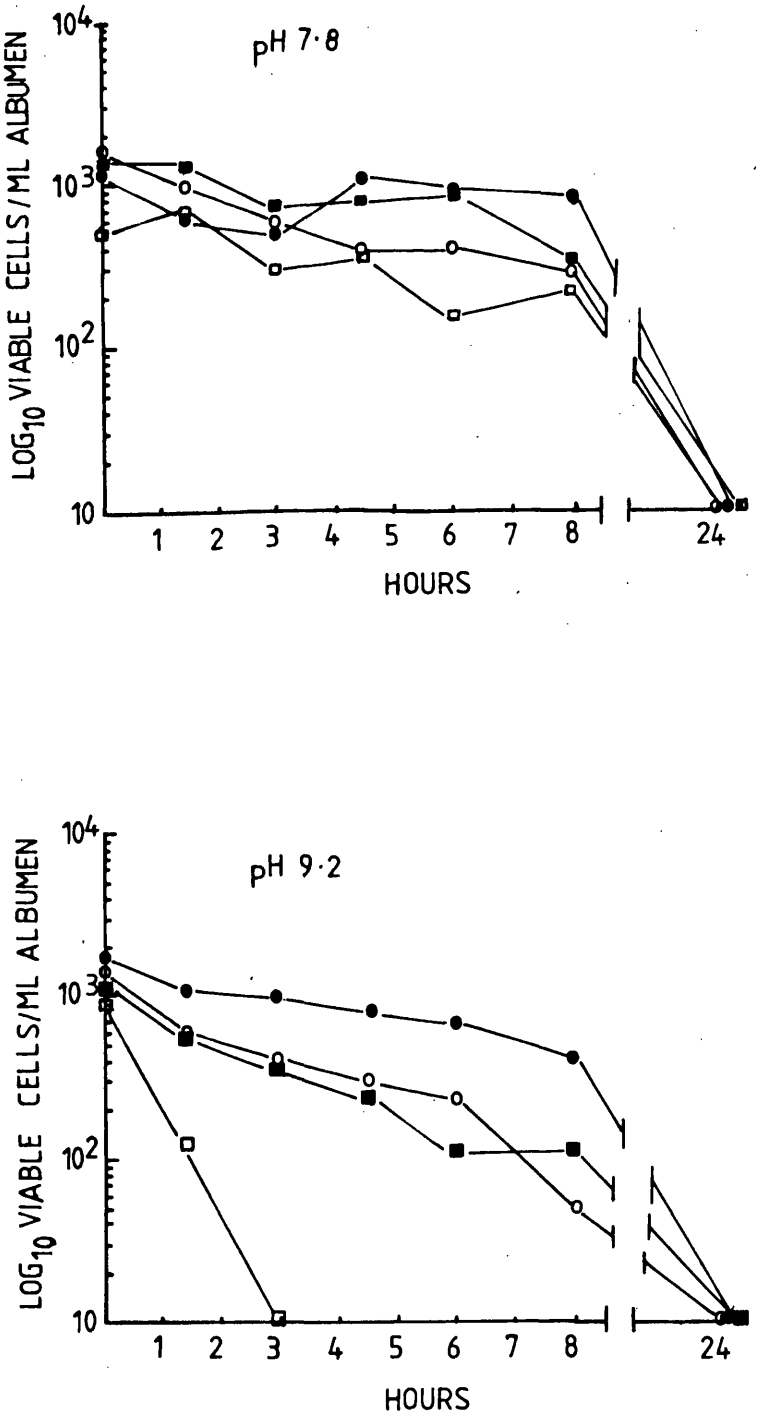

Fig. 39. The effect of incubation temperature (o, ● 30°C □, ■ 39.5°C) and pH on the fate of Bretanomyces anomalus in hen egg white (open symbols) and hen egg white saturated with iron (closed symbols).

The influence of incubation conditions on the fate of bacterial vegetative cells in hen egg albumen

(i) Physiological state of inoculum

Micro-organisms in hen egg albumen are exposed to several inimical factors therefore it would seem likely that the physiological state of the micro-organism preceeding inoculation is an important factor to consider also. Micro-organisms stressed in any way before inoculation would be expected to have little chance to survive and grow when they were actually present in the albumen. Bacteria may be stressed in a number of ways such as growth under highly acidic or basic conditions, cultures in late exponential phase or growth at sub-optimal temperatures and nutrient levels. The effect of inoculum age (Fig. 40) and inoculum growth medium (Fig. 41) were examined for cells of *E. coli* C20 inoculated into hen egg albumen. In the case of the former, there was no appreciable difference between a young culture (2.5h) and a culture well into exponential phase (18h) when these cells were inoculated into albumen. With the latter, cells were grown (18h) in nutrient broth or 'plate count broth' before inoculation into albumen. Plate count 'broth' is a medium nutritionally similar to nutrient broth but with added glucose so that cells grown in this medium should have all the enzymes necessary for the utilization of glucose 'switched on' before inoculation into albumen. Although the albumen's content of free carbohydrate is almost entirely (98%) glucose, cells grown in this medium did not appear to have any advantage over those grown in nutrient broth. In practice, the former were killed faster than those in the glucose-poor medium.

(ii) Conditions of enumeration

If, as is to be expected from the reviews by Mossel and Corry (1977) and Beuchat (1978), that the degree of injury among populations

of damaged cells varies considerably, it is essential that enumeration methods do not cause further stress to sublethally damaged cells. It is necessary, therefore, to provide the survivors of a stressed population (both lethally and sublethally damaged) with optimum conditions for recovery and growth. These conditions may be provided by, for example, growth on simple (minimal) medium or complex medium, longer periods of incubation or lower temperatures of incubation. Bacteria inoculated in egg albumen can be expected to be exposed to many stressful factors and the task of finding optimum conditions for resuscitating damaged cells would not be an easy one. In this study the effect of diluent (Table 17), plating medium (Table 18) and temperature of incubation (Table 19) on the recovery of bacteria from hen egg white was examined. Also included in these results are others obtained when two different methods of homogenisation were used in the preparation of serial dilutions (Table 20). Of the factors examined only the mixing of the albumen and diluent had an appreciable influence on the enumeration of viable cells from the albumen. This was probably not due to a chemical factor but rather the viscous nature of egg albumen which prevented a random distribution of the organisms present (Kilsby and Pugh, 1981). As the trend of results and the overall result were the same using both methods of homogenisation, method B (Table 20) was discarded in favour of the less time consuming method A.

(iii) Incubation temperature

The fate of Gram-negative bacteria in hen egg white was markedly affected by incubation temperature (Fig. 42). Temperatures of 25 and 30°C resulted initially in bacteriostasis of Gram-negative bacteria in albumen unsupplemented with iron. Growth sometimes occurred after a few days of incubation. Hen egg white changed from being bacteriostatic

to bactericidal at higher temperatures, the greatest effect occurring at 39.5°C or above i.e. at or around the body temperature of the hen. Indeed at 44°C the albumen's effect on Gram-negative bacteria closely resembled its action on Gram-positive bacteria at lower temperatures. Moreover, at 44°C the addition of casamino acids or iron sufficient to saturate the ovotransferrin had no appreciable influence (Fig. 43).

An interpretation that the curves obtained at the high temperatures resulted from 'shocking' due to transfer of cultures grown on a rich medium to the minimal conditions existing in egg albumen was discounted by the results given in Fig. 44. A death phase similar to that noted in Fig. 41 occurred in inoculated albumen that was incubated for 12h at 25°C before transfer to 37 and 39.5°C.

(iv) Hydrogen ion concentration

The pH of hen egg white determined the behaviour of Gram-negative bacteria in the same. Egg white was markedly bactericidal at pH 8.5 - 9.5 whereas at pH 8.0 or less the rate of death was diminished. Indeed in many cases bacteriostasis was evident (Figs. 45 and 46). An alkaline reaction had a notable effect on the growth of the micro-organisms even when iron was present in amount sufficient to saturate the content of ovotransferrin. A lag phase was observed with both E. coli 0141 and E. coli 0111 at pH 9.0 compared with immediate growth at pH 7.2, furthermore the final population in each case was lower at the higher pH.

In an attempt to mimic the effects observed with egg white of different pH values, E. coli 0141 was inoculated into a nutritionally simple medium (M9) buffered at different values over the range pH 7 - 9. In one series of experiments the medium was rendered iron-deficient by

extraction with 8 - hydroxyquinoline. It is evident from the results presented in Figs. 47 and 48 that there was a small interaction between pH and the iron content of the defined medium. The increase in pH alone resulted in a decrease in the overall growth of the micro-organism but this trend was accentuated by depleting the iron content of the medium. This caused a protracted lag phase and a decrease in the final population as measured by a lower absorbance. Although little growth occurred in iron-deficient medium (Fig. 48) having alkaline pH values, the medium did not give results directly comparable with those obtained with egg white. These observations may be taken as evidence that factors other than pH and iron deficiency are contributing to the antimicrobial properties of egg white but such a conclusion would be premature because of the lack of direct evidence that the minimal medium greatly resembled egg white. Nevertheless the inter-relationship of incubation temperature, pH and iron status in the antibacterial action of hen egg albumen (Figs. 49 and 50) does direct attention at previous work. Through giving emphasis to one factor only most workers succeeded in over-simplifying the antimicrobial action of egg albumen.

The relationship between incubation temperature and the iron content of the growth medium has been commented upon by Garibaldi (1971 ; 1972) who suggested that the synthesis of microbial iron-transport compounds (siderophores) secreted in response to low iron concentrations was affected at higher growth temperatures, a phenomenon that has also been observed with bacteria in mammalian sera (Kochan, 1977).

TABLE 17

THE EFFECT OF DIFFERENT DILUENTS ON THE VIABLE COUNTS OBTAINED FROM HEN EGG WHITE AT 39.5°C INOCULATED WITH ESCHERICHIA COLI C20

	Viable Counts / ml albumen *		
Time(h)	Ringers Buffer	Distilled Water	0.65% NaCl
0	1.53×10^7	1.94×10^7	1.65×10^7
1	4.10×10^6	5.85×10^6	3.16×10^6
2	6.39×10^5	2.92×10^6	5.78×10^5
3	3.27×10^5	5.23×10^5	1.44×10^5
4	2.61×10^5	2.93×10^5	1.67×10^5
5	1.53×10^5	3.56×10^4	3.66×10^4
6	5.31×10^4	2.15×10^4	3.10×10^4
7	2.6×10^4	1.3×10^4	1.95×10^4

*Mean count from 2 x 8 replicates on nutrient agar

TABLE 18

THE EFFECT OF FOUR DIFFERENT TYPES OF MEDIA ON THE VIABLE COUNTS OBTAINED FROM HEN EGG WHITE AT 39.5°C INOCULATED WITH ESCHERICHIA COLI C20

	Viable counts / ml albumen *			
Time (h)	Nutrient Agar	Plate Count Agar	Tryptone Soya Agar	M9 Minimal Agar
0	1.3×10^6	1.2×10^6	1.35×10^6	1.3×10^6
1.0	1.3×10^5	1.35×10^5	1.3×10^5	1.4×10^5
2.5	2.65×10^4	2.5×10^4	2.55×10^4	2.35×10^4
5.0	5.0×10^3	5.5×10^3	8.5×10^3	5.0×10^3
7.0	5.05×10^2	3.0×10^2	6.0×10^2	9.8×10^2
9.0	2.45×10^2	2.0×10^2	2.1×10^2	2.1×10^2
10.0	1.0×10^2	1.0×10^2	1.2×10^2	1.1×10^2

* Mean counts from 2 x 8 replicates

TABLE 19

THE EFFECT OF INCUBATION TEMPERATURE ON VIABLE COUNTS OBTAINED FROM HEN EGG WHITE AT 39.5°C INOCULATED WITH ESCHERICHIA COLI C20

Time (h)	Viable counts / ml albumen *			
	Plates incubated at 37°C for 48h		Plates incubated at 25°C for 24h then at 37°C for 24h	
	A	B	A	B
0	1.76×10^6	1.70×10^6	1.76×10^6	1.70×10^6
1	1.25×10^5	5.63×10^4	1.33×10^5	5.0×10^4
2.5	2.45×10^4	1.97×10^4	1.88×10^4	1.32×10^4
4.0	7.50×10^2	1.21×10^3	8.50×10^2	1.32×10^2
5.5	1.36×10^2	2.30×10^2	1.25×10^2	1.67×10^2
7.0	1.00×10^2	1.50×10^2	0.50×10^2	3.00×10^2
9.5	-	-	-	-

* Mean counts from 2 x 8 replicates on nutrient agar

A and B are duplicate samples

TABLE 20

THE EFFECT OF DIFFERENT METHODS OF HOMOGENISATION ON VIABLE COUNTS OBTAINED FROM HEN EGG WHITE AT 30°C AND 39.5°C INOCULATED WITH ESCHERICHIA COLI C20

Time (h)	Viable counts / ml albumen *			
	30°C		39.5°C	
	A	B	A	B
0	1.39×10^6	1.42×10^6	1.41×10^6	1.40×10^6
1.5	5.92×10^5	5.10×10^5	1.38×10^5	4.77×10^5
3	6.08×10^5	4.95×10^5	2.81×10^4	4.11×10^4
5	5.92×10^5	4.86×10^5	3.46×10^4	2.91×10^4
8	4.62×10^5	4.72×10^5	1.70×10^4	8.2×10^3
10	5.05×10^5	4.95×10^5	2.65×10^3	4.22×10^3

* Mean count from 2 x 8 replicates

A. 1ml albumen added to 9ml Ringers buffer and homogenised as in Materials and Methods.

B. 1ml albumen added to 9ml Ringers buffer and homogenised using an M.S.E. homogeniser for 2mins at full speed before diluting.

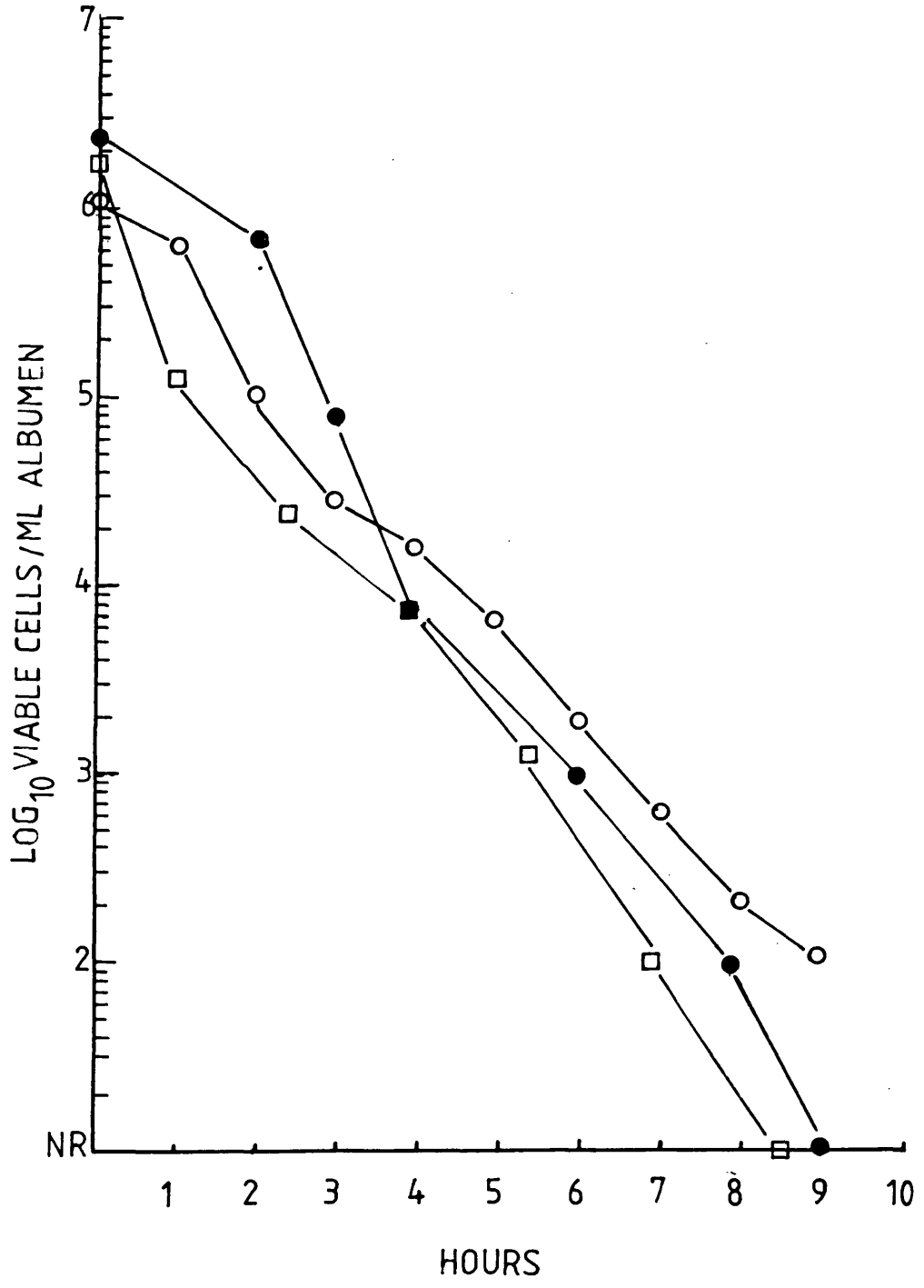

Fig. 40. The influence of inoculum age on the death of Escherichia coli C20 in hen egg white at 39.5°C. o, 2.5h; •, 12h; □, 18h.

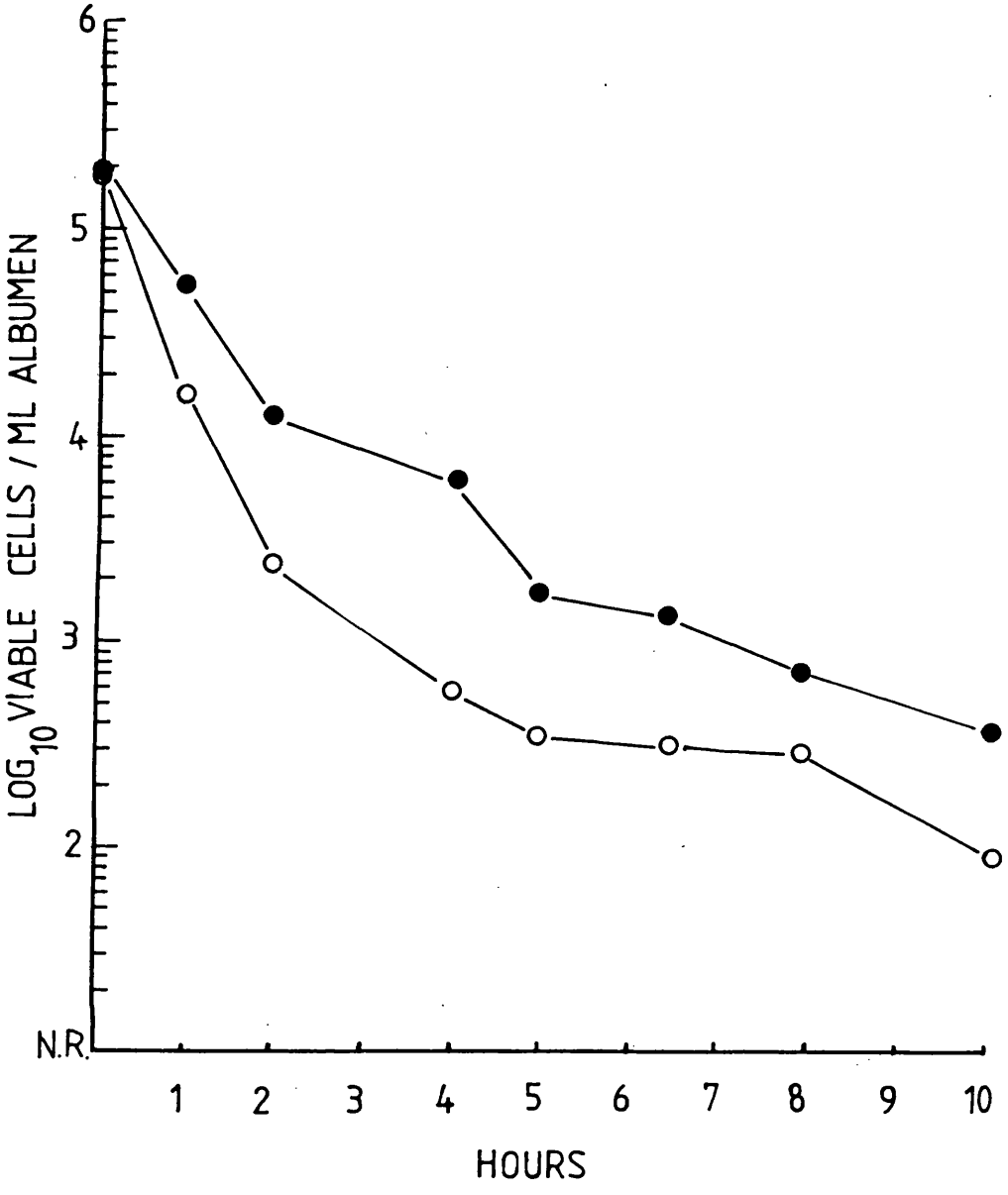

Fig. 41. The influence of inoculum growth medium on the death of Escherichia coli C20 in hen egg white at 39.5°C. o, plate count broth ; •, nutrient broth.

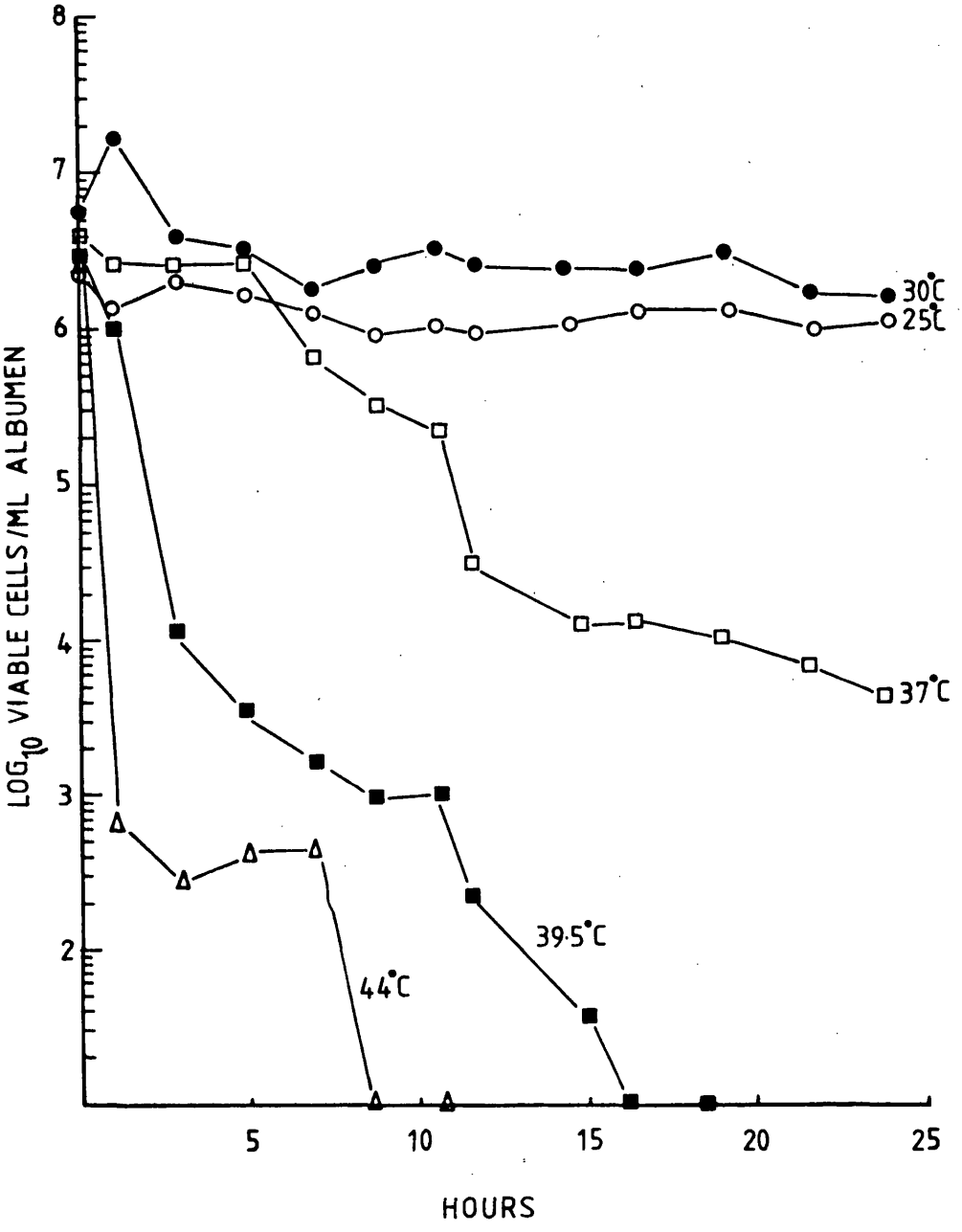

Fig. 42. The effect of incubation temperature on the fate of Escherichia coli C3650 in hen egg white.

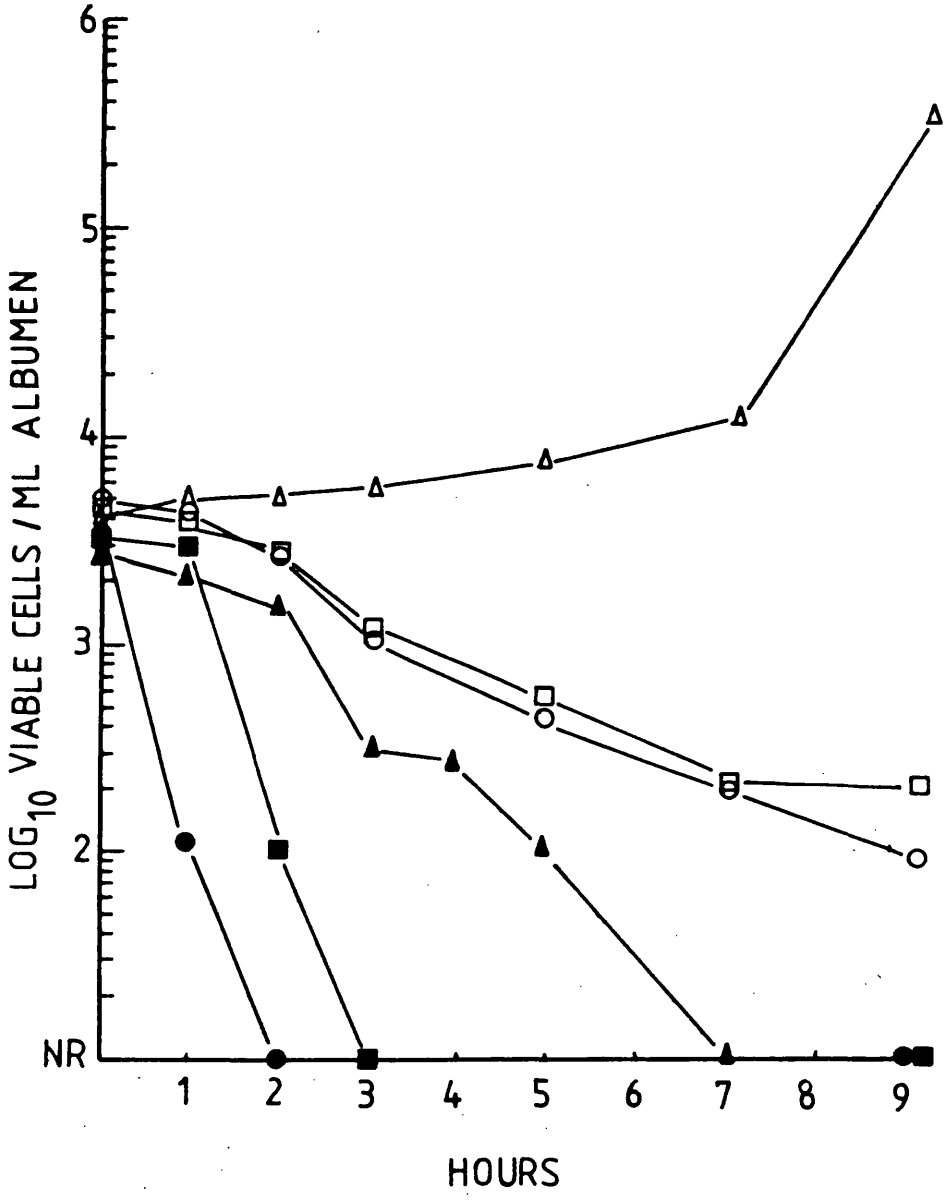

Fig. 43. The effect of 1mgml^{-1} casamino acids (□ , ■) and iron saturation (Δ , ▲) on the fate of Escherichia coli C3650 in hen egg white (o, ●) at 39.5°C (open symbols) and 44°C (closed symbols).

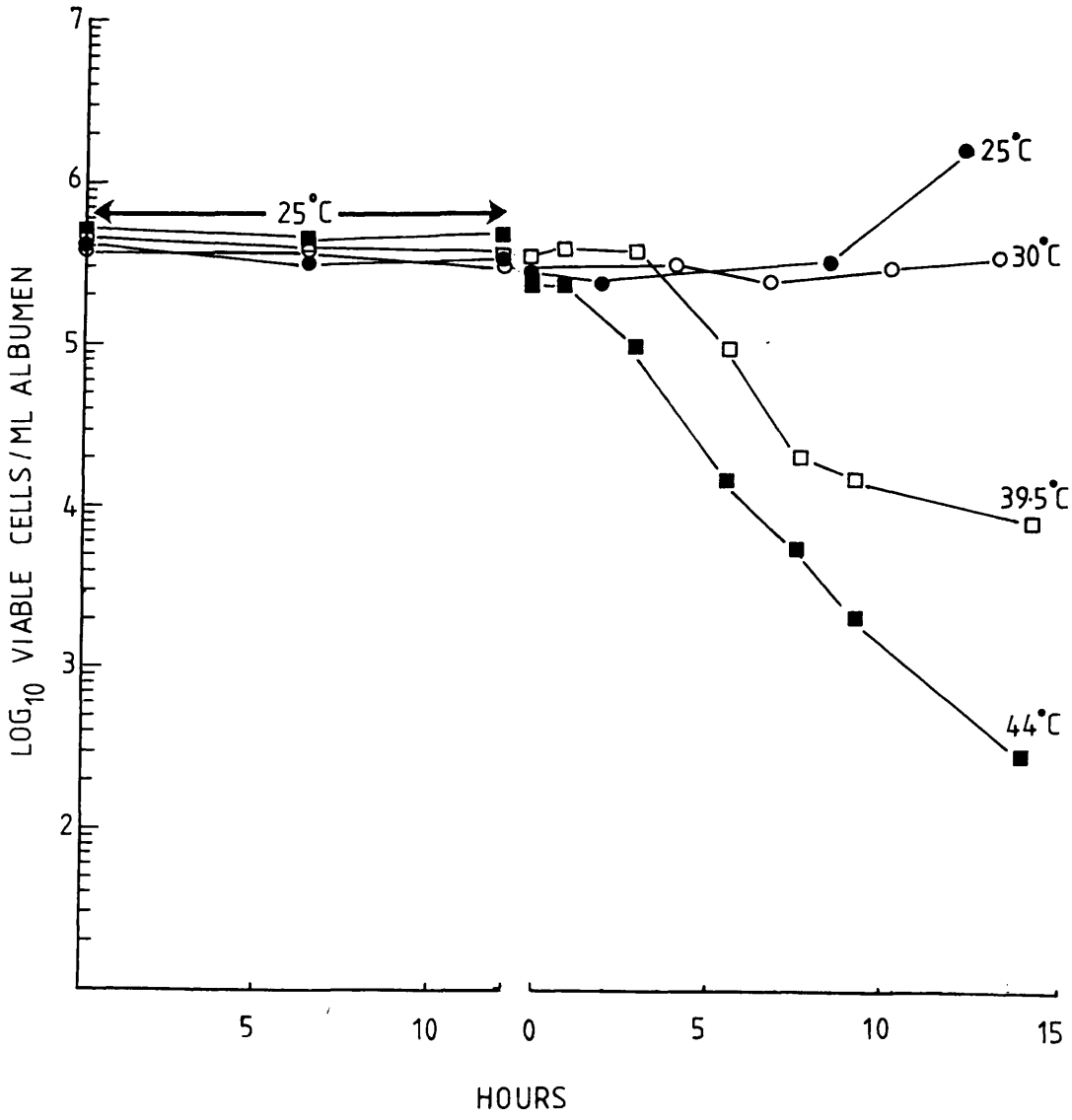

Fig. 44. The effect of incubation temperature on the fate of Escherichia coli C3650 in hen egg white after holding the inoculated white at 25°C for 12h before changing the temperature.

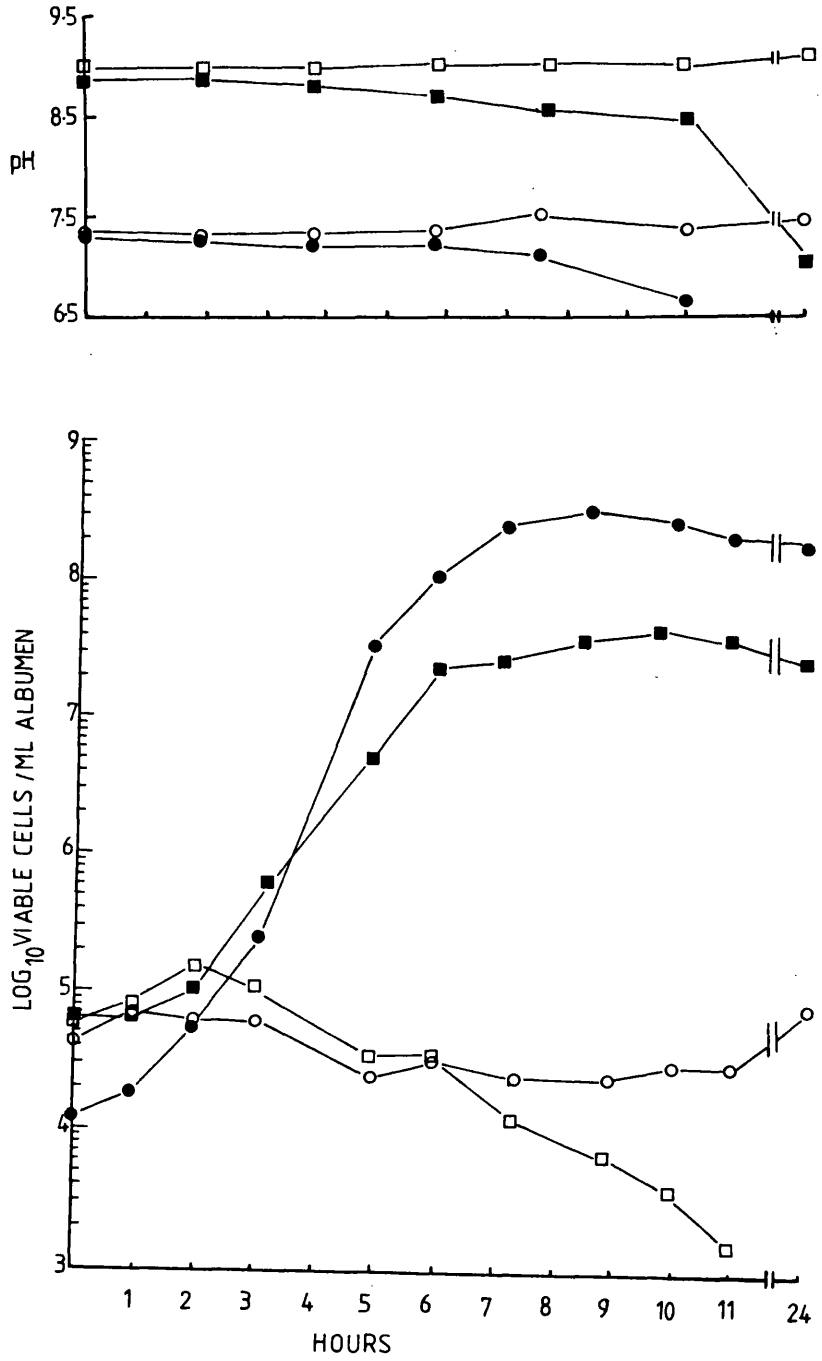

Fig. 45. The effect of egg white pH on the fate of Escherichia coli 0141 in hen egg white (open symbols) and iron saturated egg white (closed symbols) at 39.5°C.

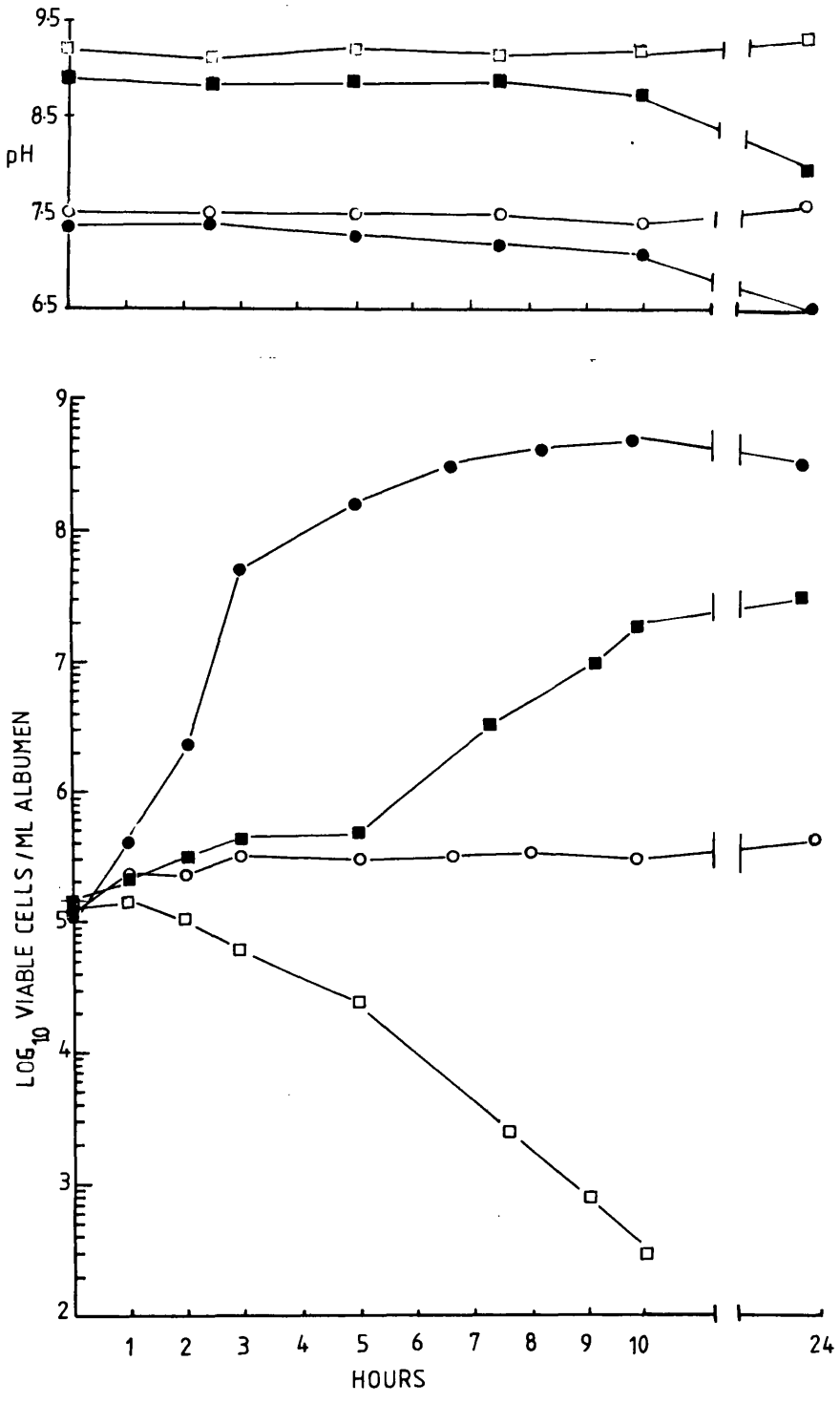

Fig. 46. The effect of egg white pH on the fate of Escherichia coli 0111 in hen egg white (open symbols) and iron saturated egg white (closed symbols) at 39.5°C.

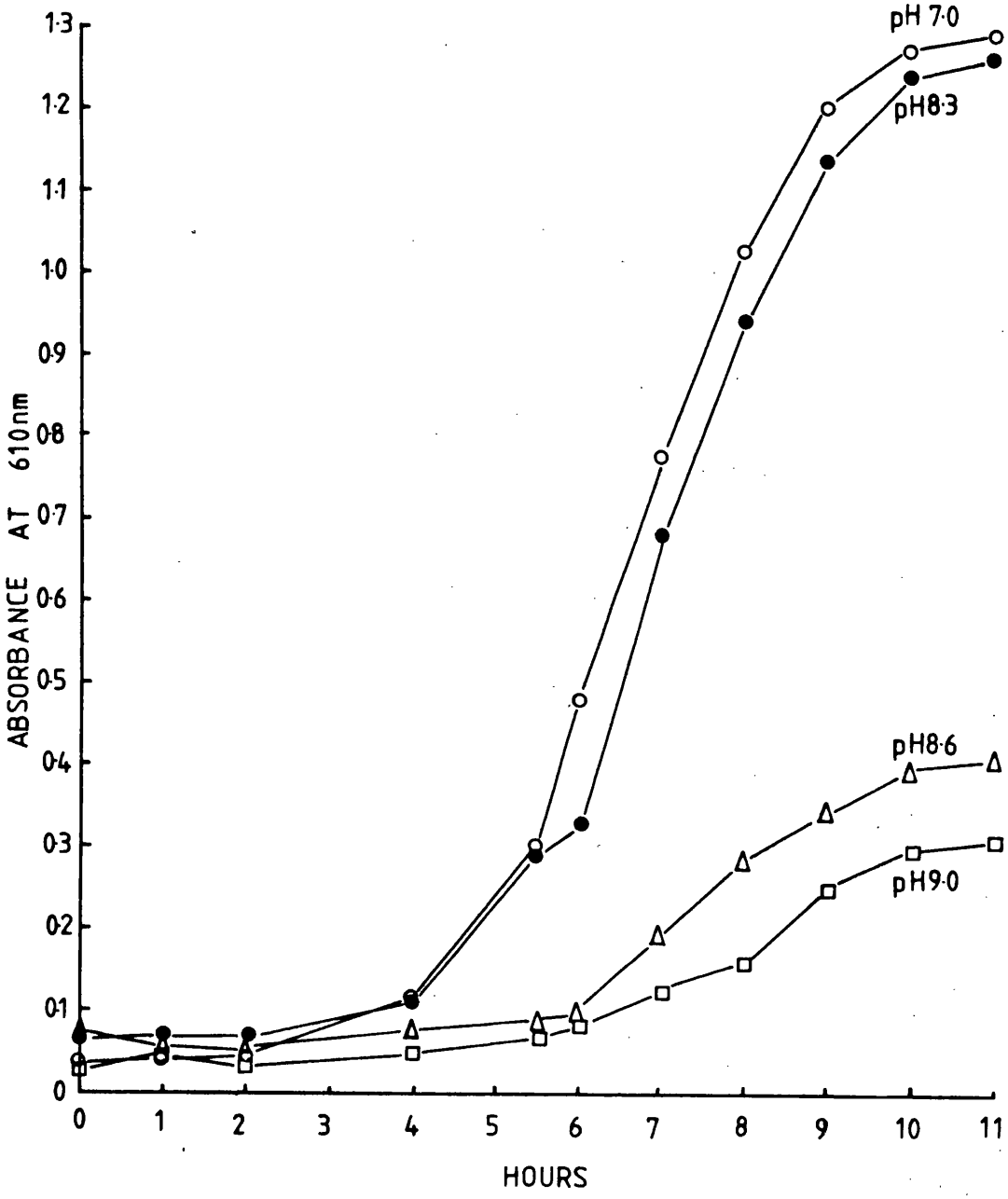

Fig. 47. The effect of pH on the growth of Escherichia coli 0141 in M9 minimal medium. The iron concentration was 2.7 $\mu g ml^{-1}$.

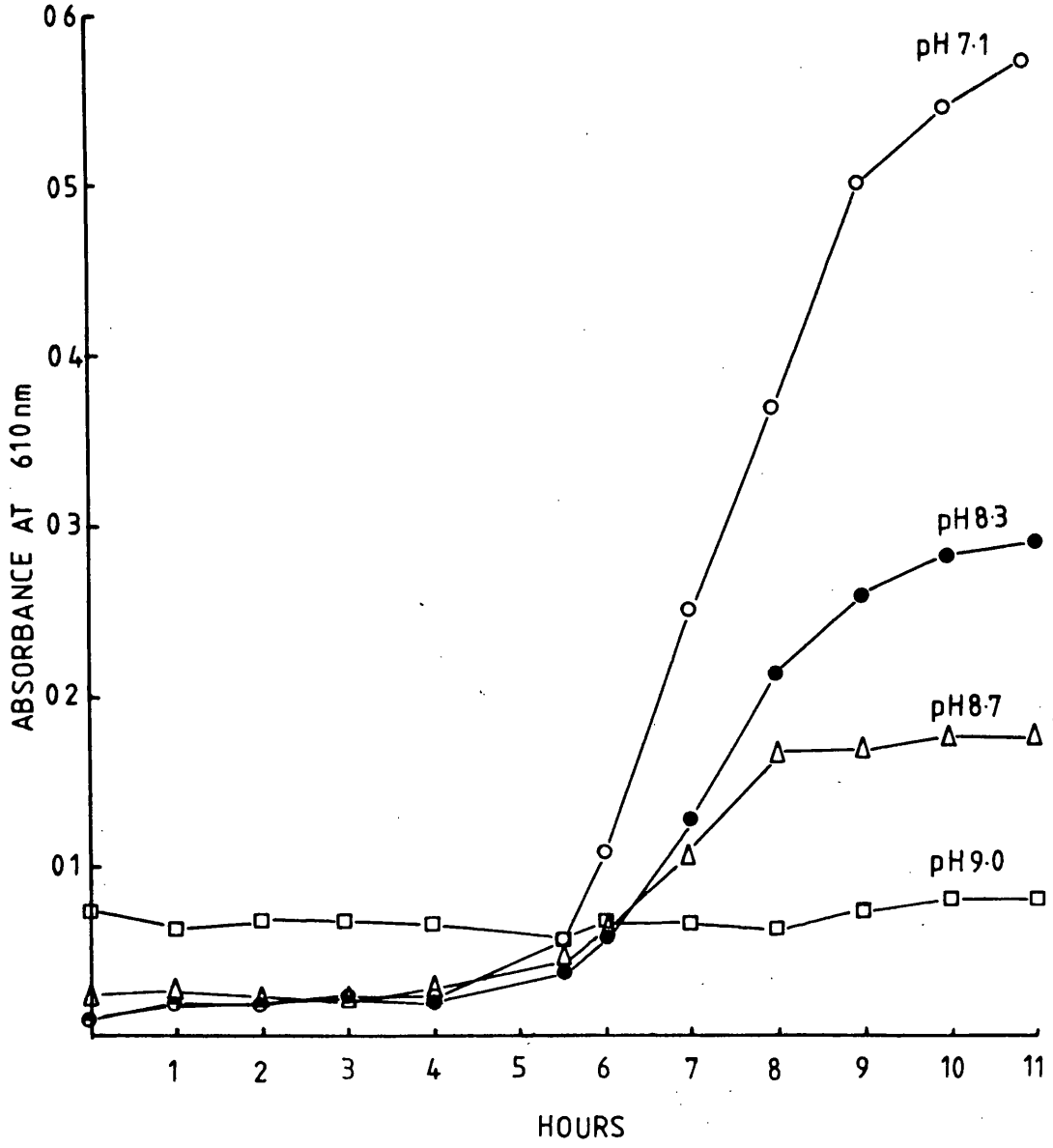

Fig. 48. The effect of pH on the growth of Escherichia coli 0141 in deironised M9 minimal medium. The iron concentration was 0.05 $\mu g ml^{-1}$.

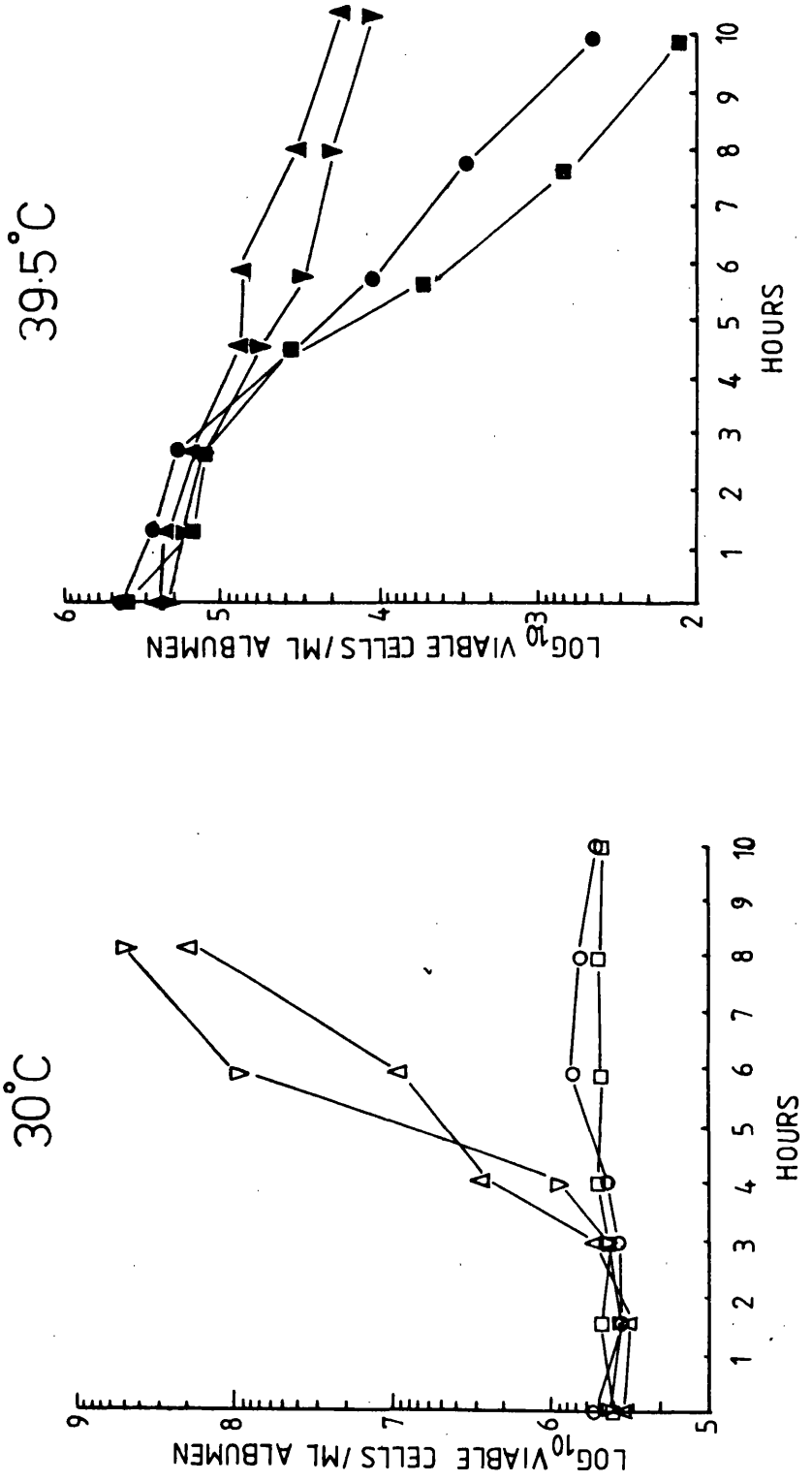

Fig. 49. The interrelationship of incubation temperature, pH and iron status on the fate of Serratia marscens in hen egg white. ○, ●, pH 7.6; □, ■ pH 9.3; △, ▲, pH 8.9; ▽, ▼, pH 7.7. Triangles, iron-saturated egg white.

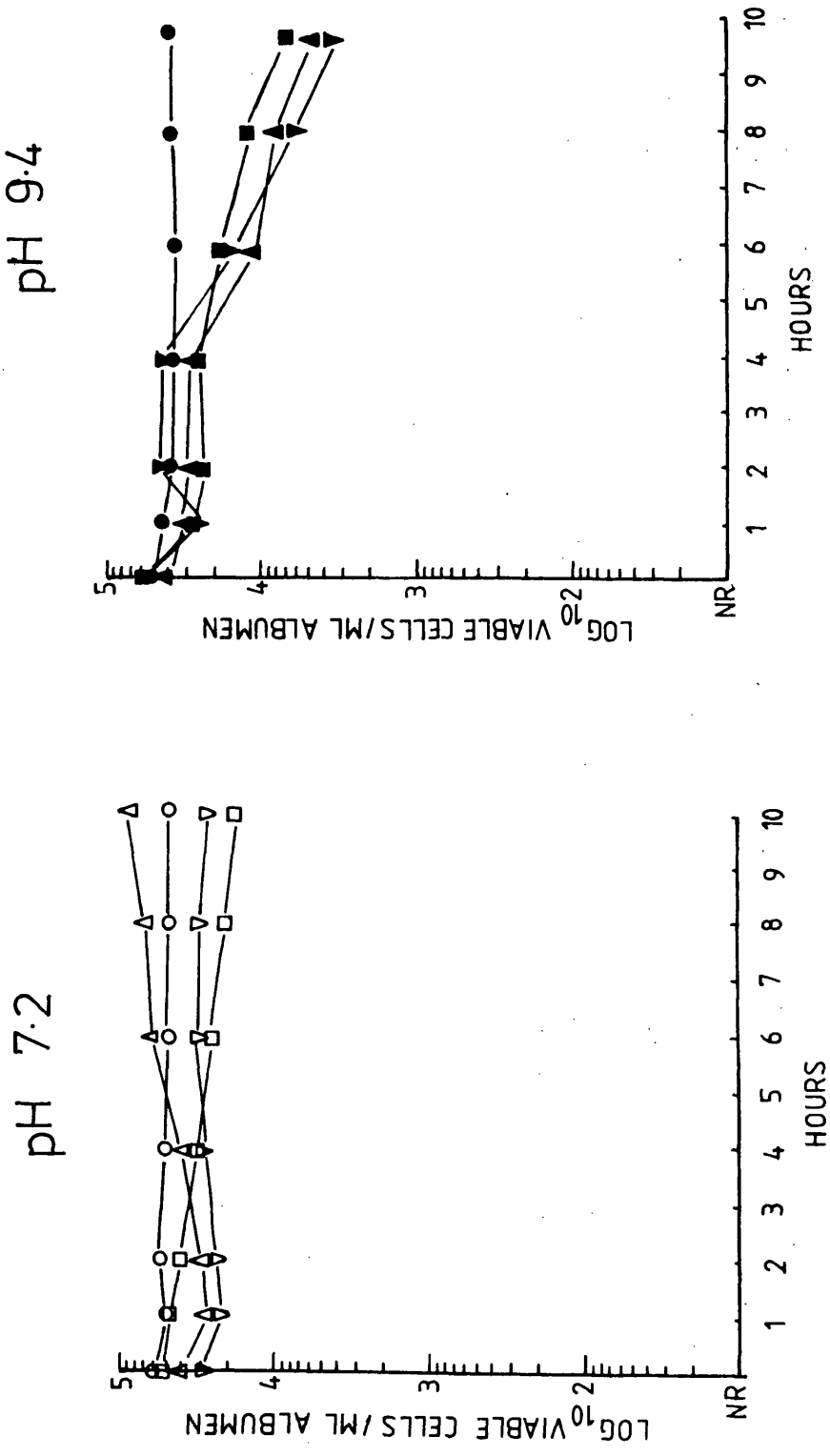

Fig. 50. The interrelationship of incubation temperature and pH on the fate of Pseudomonas fluorescens in hen egg white; o,● 4°C; □ ,■ 10°C; Δ ,▲ 25°C; ∇ ,▼ 30°C.

MICROBIAL SIDEROPHORES

When iron is present at concentrations too low to satisfy the low-affinity membrane-bound iron transport system in Escherichia coli this micro-organism, together with other members of the Enterobacteriaceae, may synthesize and secrete siderophores which solubilize exogenous iron thereby making it available for transport into the cell. The most thoroughly studied catechol siderophore is enterobactin, a cyclic trimer of 2,3-dihydroxybenzoylserine. This molecule may be hydrolysed by the enzyme enterobactin esterase into its constituent monomer molecules including 2,3-dihydroxybenzoylserine and 2,3-dihydroxybenzoic acid. The biosynthetic pathway leading to the formation of the latter named compound has been well studied using mutants of E. coli defective in its synthesis. The pathway is known to arise from chorismate the major branch compound in aromatic amino acid biosynthesis.

(i) Catechol-like compounds

The determination of enterobactin is dependant upon its extraction and purification into organic solvents. Because of the difficulty encountered when trying to isolate this compound from egg albumen an assay of one of its monomer constituent molecules 2,3-dihydroxybenzoic acid was done.

Although E. coli 0141 formed small populations in iron-extracted M9 minimal medium the level of growth exhibited by those grown in iron-sufficient M9 medium (Fig. 51). The cells in the former exhibited a longer lag period than those in the iron-sufficient medium. The protracted lag phase was also associated with the production of extracellular catechol-like compounds. The production of catechols by the iron-sufficient cells was below detectable levels ($<1\mu g$ DHBA

equivalents / ml). Catechols were not detected in inoculated albumen with or without the addition of iron (Fig. 52).

(ii) Enterobactin

The addition of enterobactin, purified from Escherichia coli AN263, supported the growth of E. coli 0141 in hen egg albumen at pH 7.9 (Fig. 53) but not at pH 9.4 (Fig. 54). Although growth occurred, it was not comparable to that achieved in previous results obtained when iron was present at concentrations needed to saturate the ovotransferrin. There was no growth at either inoculum level in the absence of enterobactin.

(iii) Enterobactin hydrolysis

Non-enzymatic hydrolysis of enterobactin may occur under conditions of high acidity or alkalinity. After 1h in Tris-HCl buffer pH 7.0 enterobactin yields two minor breakdown products (A and B ; plate 17) which can be identified by two-dimensional chromatography. The R_f values (Table 21) of these products do not resemble those of normal enzymatic breakdown products from enterobactin hydrolysis. Incubation of enterobactin in Tris-HCl pH 9.0 yields two additional products (plate 18) with R_f values (Table 21) corresponding to 2,3-dihydroxybenzoylserine and 2,3-dihydroxybenzoic acid. The chromatography of enterobactin at this pH is notable for the absence of any remaining enterobactin.

(iv) 2,3-Dihydroxybenzoic acid

The breakdown products of enterobactin hydrolysis have a limited capacity to transport iron into the vegetative bacterial cell. 2,3-dihydroxybenzoic acid has been shown to be the simplest aromatic constituent of enterobactin capable of performing this function. The

addition of a commercially available preparation of 2,3-dihydroxybenzoic acid had no effect on cells of E. coli C3650 in hen egg albumen at 30 or 39.5°C (Fig. 55). The addition of casamino acids to hen egg albumen in order to supplement the amount of free nitrogen available to micro-organisms also had no effect when added together with 2,3-dihydroxybenzoic acid.

The absence of iron-sequestering molecules in egg white inoculated with E. coli 0141 and the hydrolysis of these catechol-like compounds at high pH would tend to suggest that iron deprivation may be a major contributory factor to the inability of bacteria to survive in egg albumen. Indeed the growth of this micro-organism in albumen at pH 7.9 supplemented with enterobactin supports this contention, indicating that although E. coli 0141 can not secrete its own siderophore it can still take up the molecule when it is provided in the medium.

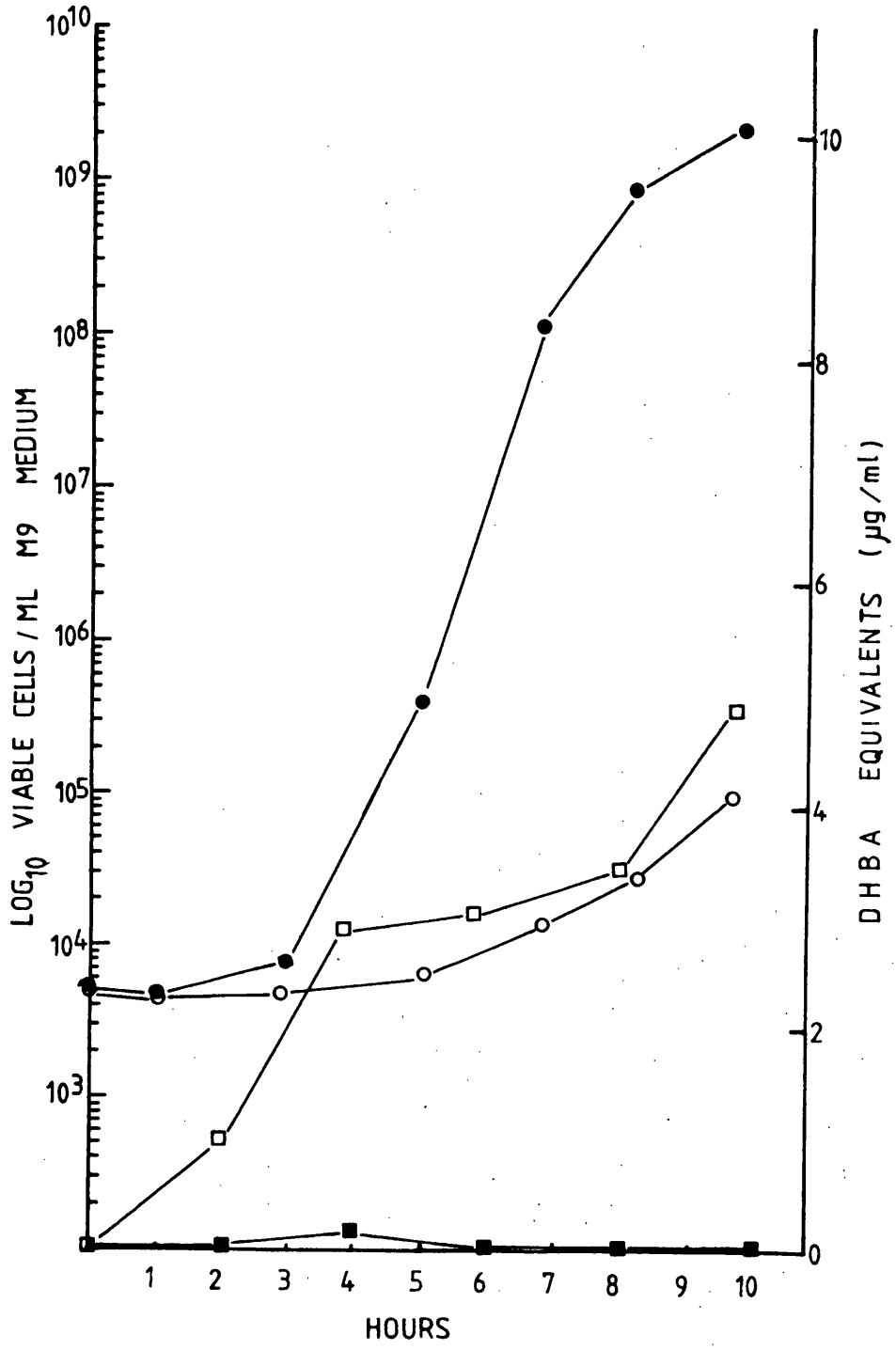

Fig. 51. The growth of Escherichia coli 0141 in iron sufficient (2.5 $\mu g ml^{-1}$) M9 minimal medium (closed symbols) and iron deficient (0.05 $\mu g ml^{-1}$) M9 minimal medium at 37°C with corresponding catechol production (squares).

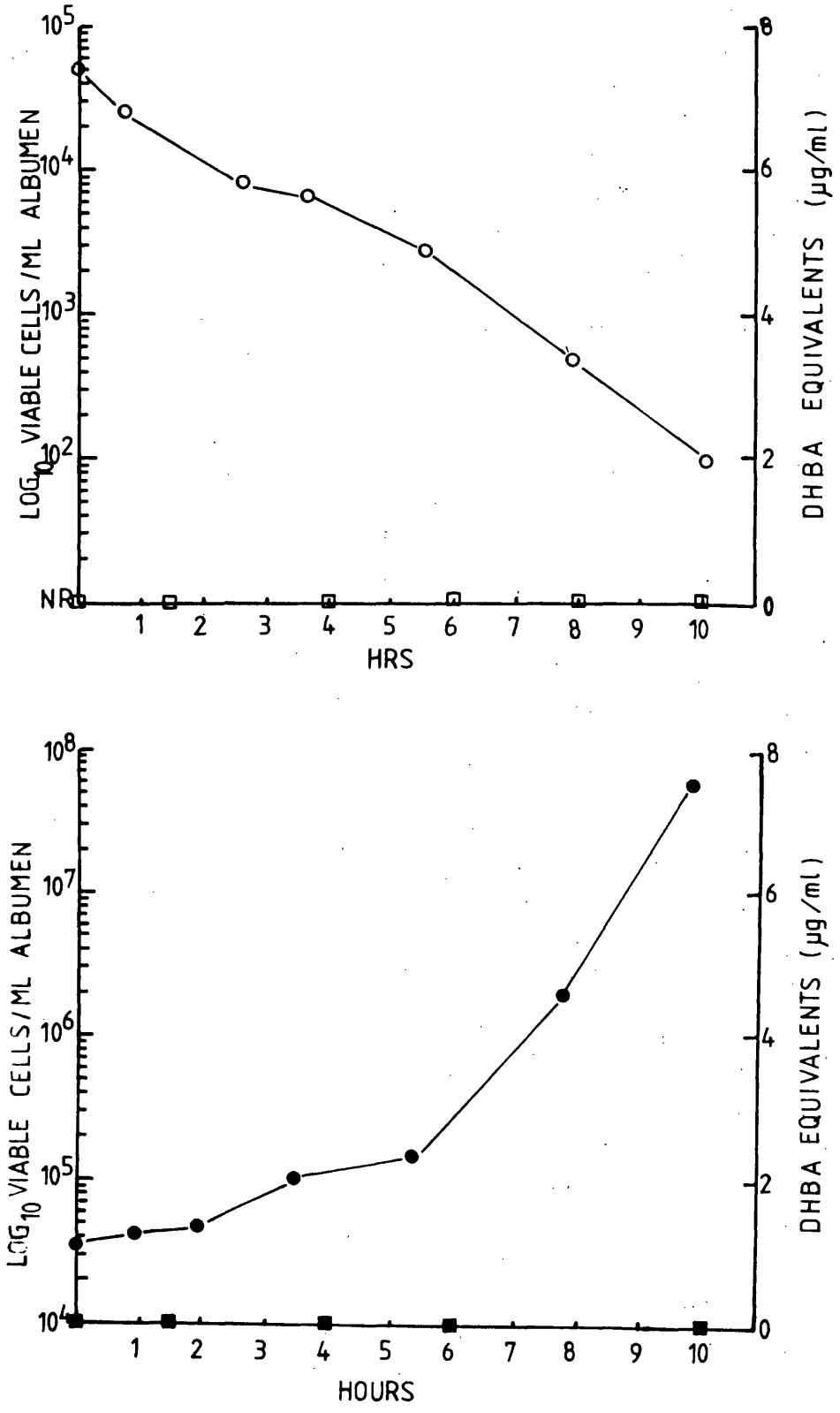

Fig. 52. The fate of Escherichia coli 0141 in hen egg white (open symbols) and iron saturated hen egg white (closed symbols) at 37°C with no corresponding catechol production (squares).

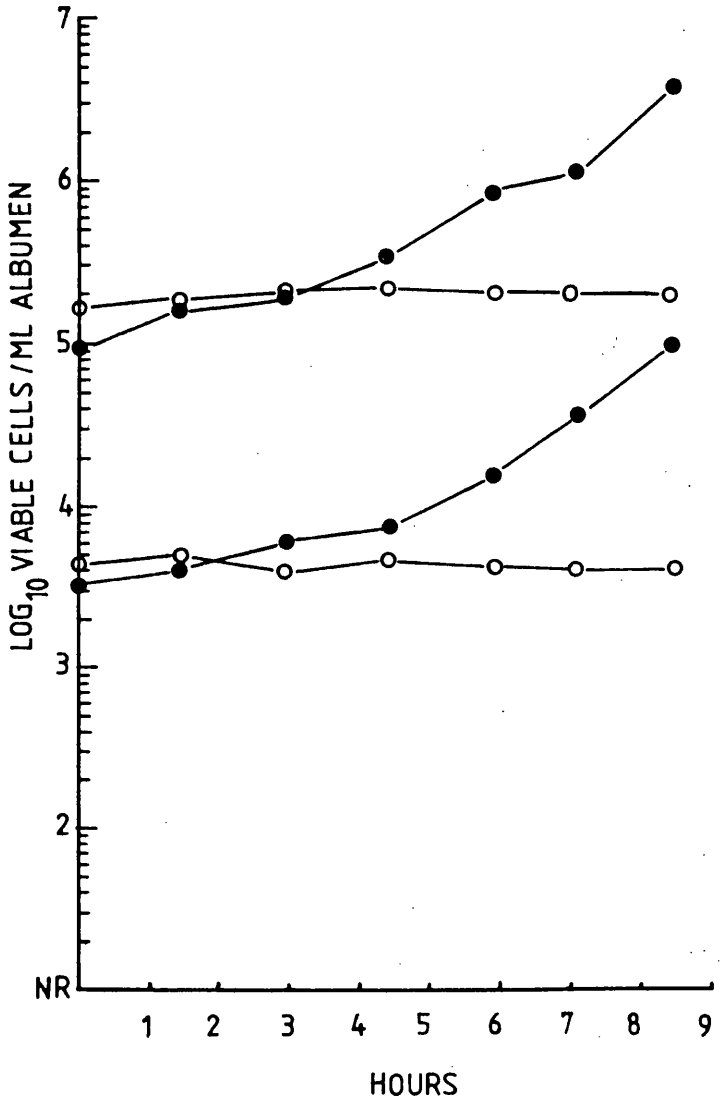

Fig. 53. The effect of adding purified enterobactin ($10 mg ml^{-1}$) on the fate of Escherichia coli 0141 in hen egg white at pH7.9 and 39.5°C. o albumen alone • albumen plus enterobactin and $2\ \mu g ml^{-1}$ iron added as $Fe(NH_4)_2(SO_4)_2.6H_2O$.

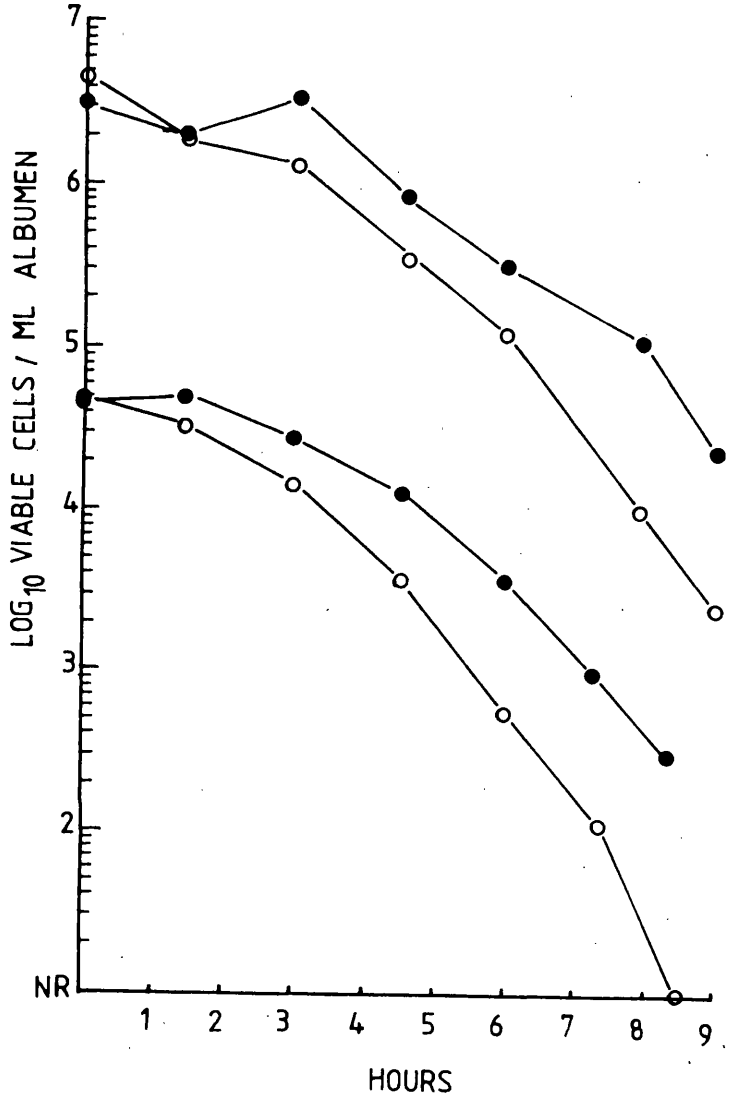

Fig. 54. The effect of adding purified enterobactin ($10 mg ml^{-1}$) on the fate of Escherichia coli 0141 in egg white at pH9.4 and 39.5°C. o albumen alone • albumen plus enterobactin and 2 $\mu g ml^{-1}$ iron added as $Fe(NH_4)_2(SO_4)_2.6H_2O$.

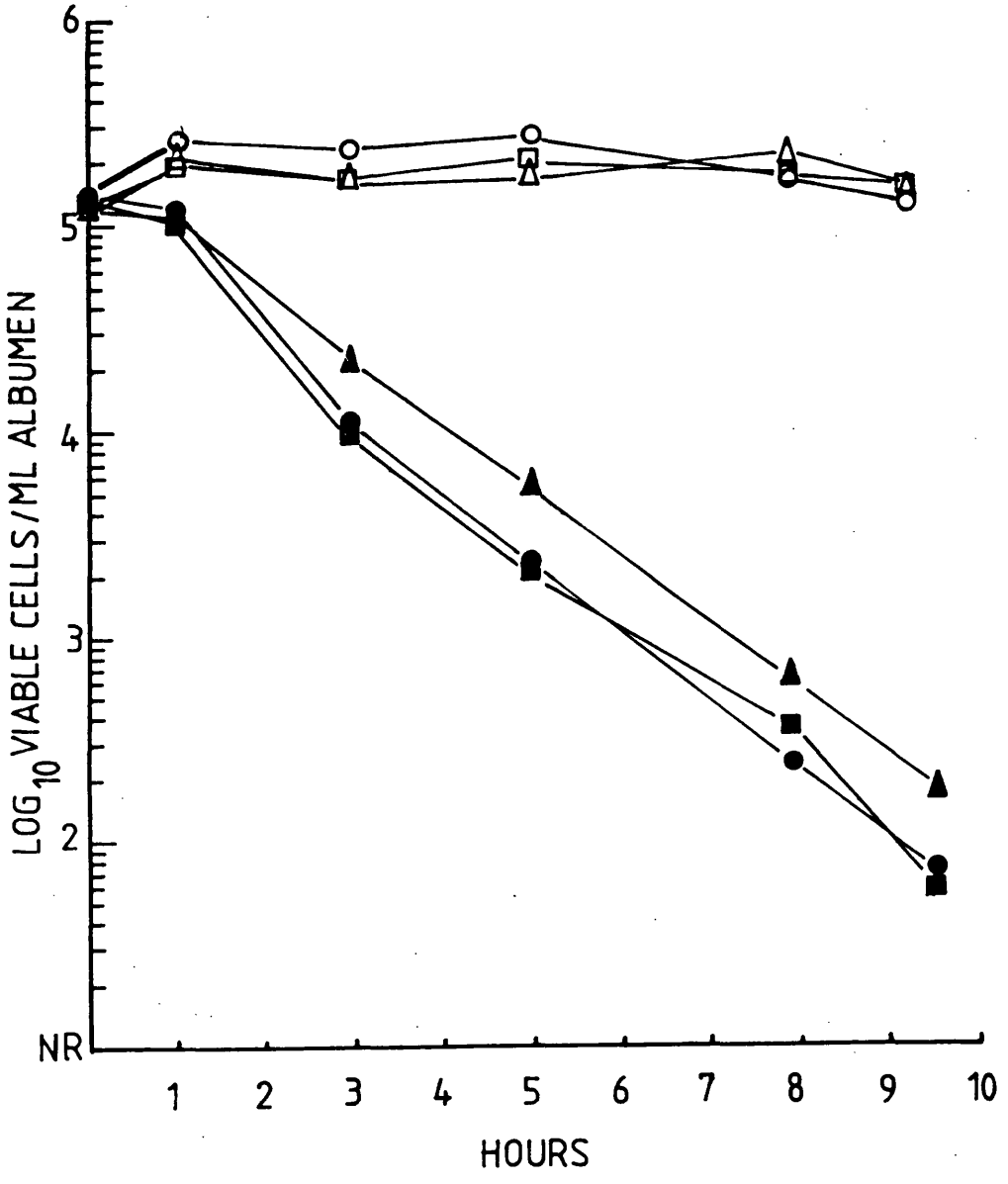

Fig. 55. The effect of a commercially available preparation of 2,3-dihydroxybenzoic acid with (Δ , ▲) or without (□ , ■) casamino acids ($1 mgml^{-1}$) on the effect of Escherichia coli C3650 in hen egg white at 30°C (open symbols) and 39.5°C (closed symbols). o, • albumen alone.

TABLE 21

Rf VALUES FOR THE COMPONENTS OF ENTEROBACTIN HYDROLYSIS ISOLATED BY THIN LAYER CHROMATOGRAPHY.

pH of Hydrolysis	Component Rf in first solvent *					Component Rf in second solvent *				
	A	B	Ent	DBA	DBS	A	B	Ent	DBA	DBS
7.0	0.57	0.43	0.43	-	-	0.69	0.43	0.04	-	-
9.0	0.35	0.36	-	0.71	0.74	0.90	0.74	-	0.71	0.008

* First solvent system = Benzene-Acetate-water

** Second solvent system = Ammonium formate-Formic Acid

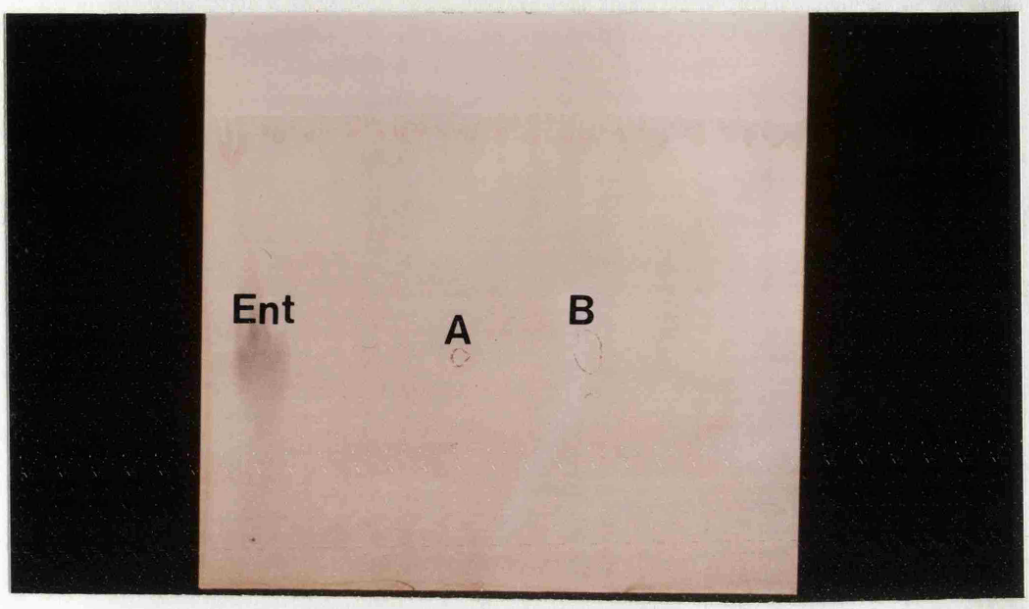

Plate 17. Two-dimensional chromatogram showing non-enzymatic breakdown of purified enterobactin (see text) at pH7.0 yielding two breakdown products (A and B). Abbreviation :Ent,enterobactin.

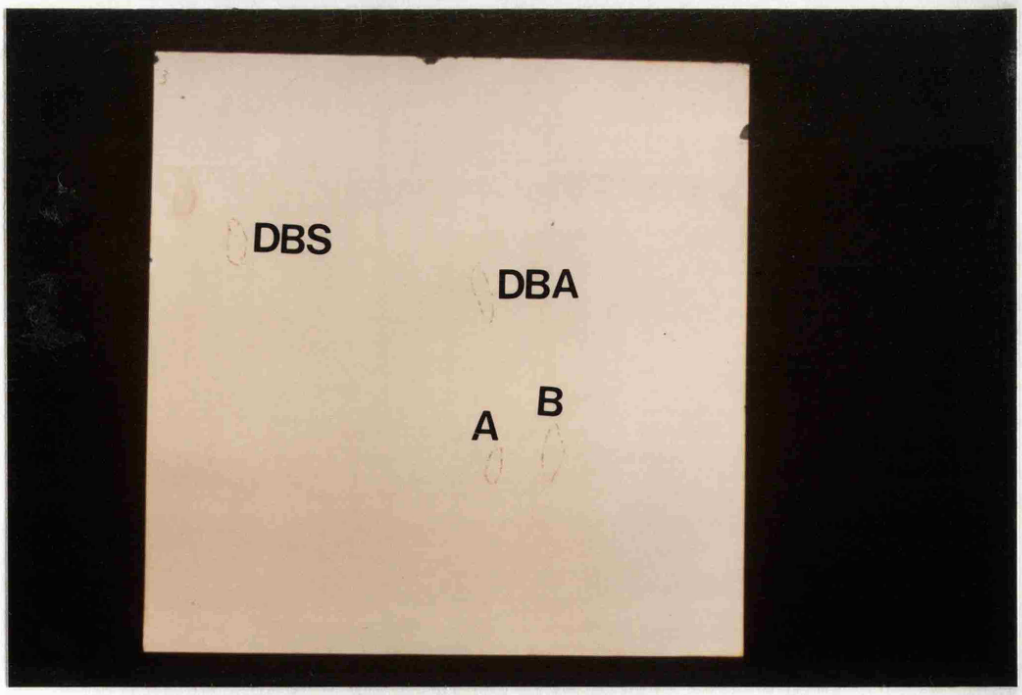

Plate 18. Two-dimensional chromatogram showing non-enzymatic breakdown of purified enterobactin (see text) at pH9.0 yielding 2,3-dihydroxybenzoylserine (DBS) and 2,3-dihydroxybenzoic acid (DBA) in addition to A and B.

A COMPARATIVE STUDY OF AVIAN EGG ALBUMEN

As far as can be ascertained, little work has been done on the antimicrobial defence of albumen other than that of the hen's egg. The opportunity was taken to study the growth of E. coli in albumen from various waterfowl eggs. The species included in this study are listed in Tables 22 and 23. As the eggs came from Slimbridge Wildfowl Trust and a detailed history was not available they were examined for microbial contamination, iron content and pH of the albumen. The results are summarized in Tables 22 and 23. Although a broad spectrum of iron concentrations was found it was noteworthy that there was no apparent correlation between the presence of bacteria and the albumen's iron content. For example, Black-headed duck albumen which had a very high iron content was sterile (Table 23) whereas Puna Teal albumen was contaminated without any detectable iron (Table 22). Of the contaminated whites several contained bacteria which gave colonies surrounded by pigment which had diffused into the plate count agar. These colonies contained tiny Gram-negative rod-shaped bacteria and were probably members of the Pseudomonad genus. It was also notable that with the exception of the Black-headed duck all the sterile whites had a pH of not less than 9.1.

The effect of egg albumen on the growth of micro-organisms is not restricted to birds of the domesticated variety especially the domestic fowl. Several of the sterile egg whites in Table 23 were tested for their ability to inhibit the growth of E. coli 0141 at 39.5°C (Figs. 56 and 57). The initial pH values ranged from 9.1 - 9.3 hence the variation that occurred between the different whites, say for example Aleutian Canada Goose and hen, can not be explained on the basis of different pH values. As so little material was available for study

it was not possible to titrate the albumen of waterfowl eggs to establish the amount of Fe^{3+} required to saturate the ovotransferrin present, a component common to all eggs (Sibley, 1960 ; 1970). As the available evidence would suggest that hen's egg albumen contains a relatively high concentration of ovotransferrin compared to most other whites, apart from some species of pheasant and quail (Osuga and Feeney, 1974 ; Clark et al., 1963 ; see Figs. 17 and 18) the same amount of iron needed to saturate hen ovotransferrin was added to waterfowl albumen which was incubated at 39.5°C. In all cases the addition of iron was sufficient to overcome the inhibition achieved by the untreated albumen (Fig. 57).

TABLE 22

THE pH AND IRON CONCENTRATION OF A RANGE OF CONTAMINATED AVIAN EGG WHITES

Avian species	Approx age of eggs	No.of eggs used	albumen pH	contamination broth 25	broth 30	broth 37°C	plate 25	plate 30	plate 37°C	iron concentration (µgml)
European goldeneye	26d	1	6.5	++	++*	++	++*	++*	++*	3.0
Mandarin duck	29d	6	9.4	++	++	++	++*	++*	++	(-)
European shelduck	21d	3	9.3	+	+	+	+*	+*	+*	1.6
Smew	19d	7	7.0	++	++	++	++*	++*	+*	6.3
Puna Teal	17d	2	7.9	+	+	+	+	+	+	(-)
Greater Snow Goose	27d	3	6.4	++	++	+	-	++*	++*	(-)
Bewick Swan	22d	2	8.6	++	++	++	++*	++*	++*	2.1
Chinese spotbill	59d	2	9.0	++	++	++	++*	++*	++*	(-)

\+ Moderate contamination

++ Gross contamination

* Pigment produced in the medium

(-) Value below the level of detection i.e. $< 0.05\ \mu g ml^{-1}$

TABLE 23

THE pH AND IRON CONCENTRATION OF A RANGE OF STERILE AVIAN EGG WHITES

Avian species	Approx age of eggs used	No. of eggs used	albumen pH	iron concentration ($\mu g ml^{-1}$)
Barrow Goldeneye	21d	2	9.1	2.4
Comb Duck	21d	8	ND	1.3
Black-headed Duck	24d	1	7.8	10.2
White-faced Whistling Duck	34d	4	9.3	3.3
South American Ruddy Duck	19d	2	9.2	2.4
North American Ruddy Duck	27d	2	9.2	1.3
Pacific White Front	29d	1	9.3	4.2
Smew	21d	2	9.2	(-)
Ringed Teal	21d	6	9.3	1.0
Puna Teal	22d	2	9.3	(-)
Emperor Goose	?	1	9.3	(-)
Red-breasted goose	21d	1	ND	(-)
Barnacle Goose	28d	2	9.2	1.0
Aleutian Canada Goose	27d	3	9.2	2.3
Black-necked Swan	27d	3	9.3	(-)
White-winged Wood Duck	54d	1	ND	5.6
Domestic Fowl	12d	6	9.1	1.0
Domestic Turkey	10d	6	9.0	(-)

ND Value not determined

(-) Value below level of detection i.e. $< 0.05 \mu g ml^{-1}$

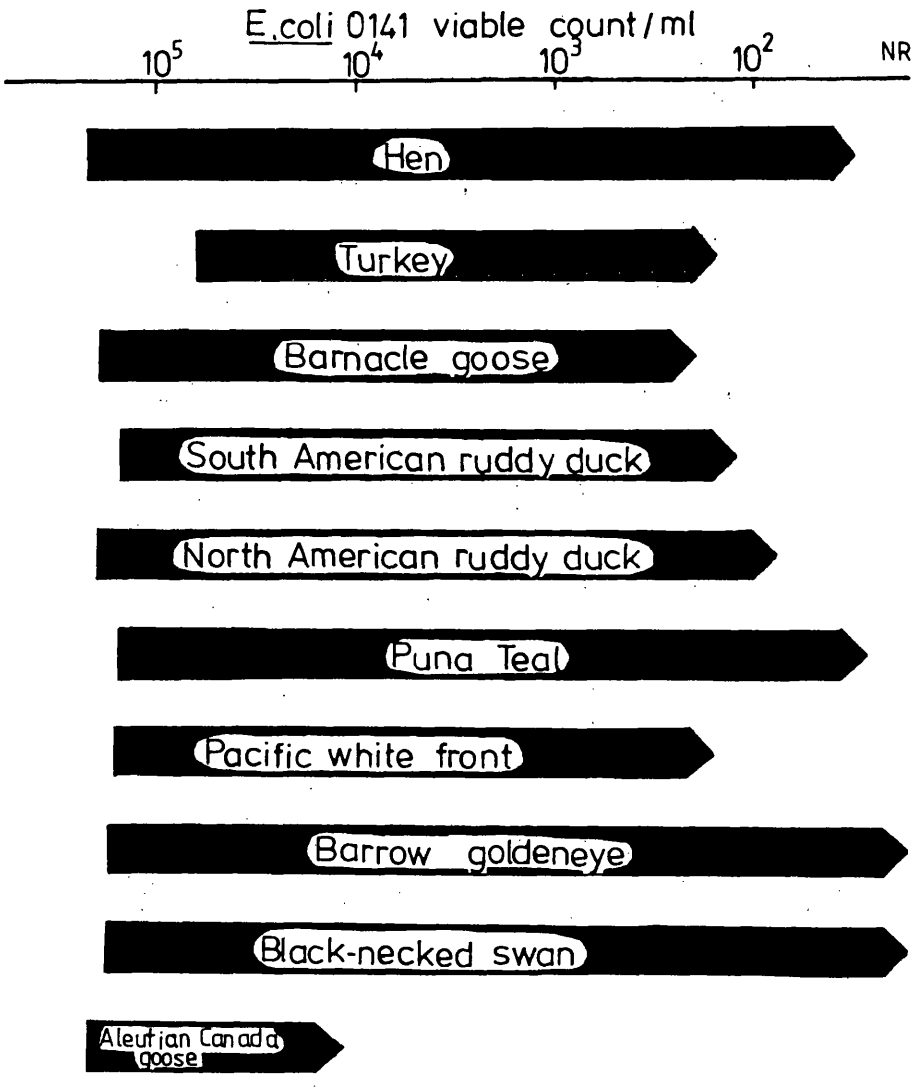

Fig. 56. The fate of Escherichia coli 0141 in a range of avian egg whites incubated at 39.5°C for 12h.

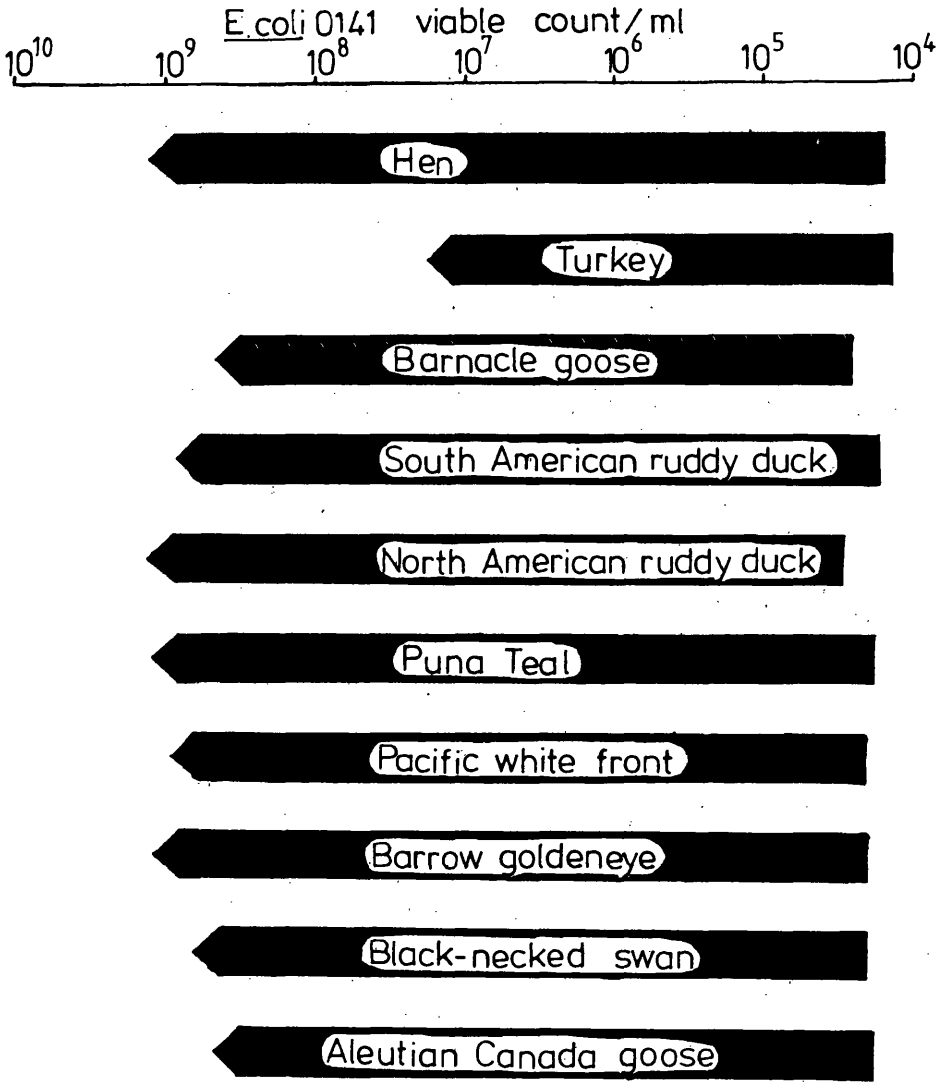

Fig. 57. The growth of Escherichia coli 0141 in 'iron-saturated' avian egg whites incubated at 39.5°C for 12h.

DISCUSSION

Of a number of trace elements required for microbial growth, the available evidence suggests that iron is the most important (Weinberg, 1966). The concentration of iron needed for growth of the commonly studied bacteria is generally between 0.3-4.0 μM (Weinberg, 1971). As standard laboratory media such as nutrient broth or tryptone soya broth contain approximately 4.0-7.0 μM iron (Weinberg, 1971), they do not need to be supplemented in order to support growth. Nevertheless all living systems are faced with the problems that at neutral pH and atmospheric tensions Fe(II) will be oxidised by molecular O_2 to the Fe(III) state. Fe(III) is, in turn, readily hydrolysed to form insoluble Fe(III) hydroxide complexes in which iron is biologically unavailable.

The equation used to describe the hydrolysis of Fe(III) :

$$Fe(III) + 3(OH)^- \longrightarrow Fe(OH)_3 \qquad (\text{Log}K_{sp} \neq 38)$$

is a gross over simplification. A compound of the composition $Fe(OH)_3$ has never been isolated. The actual hydrolysis of Fe(III) appears to proceed by a series of discrete steps each being an equilibrium process involving deprotonisation and/or condensation. The following reactions have been identified :

$$Fe(H_2O)_6^{3+} \rightleftharpoons Fe(H_2O)_5(OH)^{2+} + H^+$$

$$Fe(H_2O)_5(OH)^{2+} \rightleftharpoons Fe(H_2O)_4(OH)_2^{+} + H^+$$

$$Fe(H_2O)_4(OH)_2^{+} \rightleftharpoons Fe_2(H_2O)_8(OH)_2^{2+} + H^+$$

Subsequent steps would lead to the formation of high molecular weight products such as the spherical polymers described by Spiro and

Saltman (1969). Thus organisms dependent on O_2 have evolved specific molecules for the scavenging, transport and storage of iron in a utilizable form. For example, the synthesis of siderophores by micro-organisms is well documented. These compounds, usually simple, low molecular weight phenolates or hydroxamates, facilitate solubilization and transport of the metal into the cell from the surrounding medium. In vertebrates more complex structures have evolved such as the ferritins and transferrins which are involved respectively in the storage and transport of iron (Harrison et al., 1977 ; Zschocke and Bezkorovainy, 1974).

The total amount of iron present in biological fluids such as mammalian milk and plasma is more than sufficient for bacterial growth. Depending on the species, milk of normal healthy individuals contains between 10-30 μM and plasma, between 10-65 μM. It is well documented that this iron as well as the small amount (about 17 μM) present in hen egg white is "locked up" by the iron-binding proteins, lactoferrin, transferrin and ovotransferrin respectively (Bullen et al., 1978 ; Board and Hornsey, 1978). The actual amount of free iron in equilibrium with these proteins is only about 10^{-18}M which is far too low for normal bacterial growth. Although a specific function, namely that of iron transport (Zschocke and Bezkorovainy, 1974), can be ascribed to serum transferrin it is generally believed that an important function of all three of these proteins is the inhibition of microbial growth through the chelation of iron. When one considers the neutral or alkaline pH values of biological fluids at which the transferrins have optimal or near optimal activity, this view does not come as too much of a surprise. The ability of the host to withhold iron from micro-organisms has been accorded the term "nutritional immunity" (Kochan, 1973) and it has been suggested that the ability of the micro-organism to acquire

iron from the host and the ability of the host to deny it to the micro-organism constitutes "a battle of chelating agents" (Glynn, 1972) - transferrin versus siderophores.

Microbial inhibition by hen egg white

My results were in accord with the earlier observations (Sharp and Whitaker, 1927 ; Schade and Caroline, 1944 ; Garibaldi, 1960 ; Board and Halls, 1973c), namely that unsupplemented hen egg albumen does not support the growth of a wide range of micro-organisms. Although the older literature ascribes this property to two proteins, lysozyme and ovotransferrin, there is no evidence (Board, 1969) to support the notion that the former plays an important part in protecting table eggs during storage and distribution. With this in mind it would seem that the results in Figs. 11-13 showing the susceptibility of Gram-positive organisms to albumen contradict the last statement. Lysozyme-resistant Gram-positive bacteria such as Bacillus cereus T and Micrococcus luteus (Fig. 14) however show no apparent advantage over lysozyme-sensitive strains in hen egg albumen (Fig. 11). One interpretation of this may be that factors other than lysozyme are acting on the lysozyme resistant strains thereby inducing lysozyme sensitivity. A more likely explanation is that factors other than lysozyme are directly responsible for the death of the lysozyme-resistant bacteria. This latter explanation is partly supported by failure to render Escherichia coli O111 sensitive to lysozyme in iron deficient minimal media at high pH (Fig. 17). Vos (1964) and Zinder and Arndt (1956) have shown that in complex media lysozyme sensitivity in Gram-negative bacteria can be induced by a chelating agent and high pH. My own interpretation agrees with that of Board (1968) who added lysozyme to a mixed bacterial flora from egg shells without causing a

significant reduction in numbers. Thus the available evidence casts doubt on the cardinal role accorded to lysozyme which arose mainly from the classical observations of Fleming (1922). The predominance of a Gram-negative flora in rotten eggs (Harry, 1957 ; Pathak et al., 1960 ; Board, 1965), is probably a reflection of such organisms' simple nutritional requirements rather than lysozyme-sensitivity of Gram-positive bacteria.

The observation that hen egg albumen contains an iron-binding protein (Alderton et al., 1946), ovotransferrin (Schade and Caroline, 1944), provided the first substantial evidence to account for the failure of bacteria to grow in egg albumen. The same mechanism was later demonstrated in both plasma (Schade and Caroline, 1946) and milk (Masson et al., 1966). Many investigators have demonstrated that nutritional immunity may be overcome simply by supplementing the biological fluid in vitro or in the host with small amounts of iron. In normal human plasma, the transferrin concentration is about 30 µM and iron 18-21 µM. As the protein has the capacity to bind 60 µM of iron it is normally only 30-35% saturated with the metal. The percentage saturation of the transferrin is correlated directly with the ability of the serum to support growth of micro-organisms. In vitro tests have shown that 60-80% saturation of the serum transferrin is generally sufficient to neutralize its inhibitory effect (Schade, 1963 ; Caroline et al., 1964 ; Rogers, 1967 ; Bullen and Rogers, 1969). Pathogenic bacteria in vivo are able to multiply when given access to adequate iron. Thus in experimental infaction of mice or rats with Pseudomonas aeruginosa, Salmonella typhimurium, Lysteria monocytogenes, Klebsiella pneumoniae or Escherichia coli, the LD_{50} was reduced significantly and microbial growth enhanced by intraperitoneal, intramuscular or intravenous injection of iron (Chandlee and Fukui, 1965 ;

Martin et al., 1963 ; Sword, 1966). It is also notable in hyperferraemic patients, when the iron saturation is greater than 60% as a result of malaria, leukaemias, thalassemia etc., or in hypotransferrinemic patients, when the transferrin concentration may be as low as 3 μM and the saturation level as high as 100% as in sufferers of the disease kwashiorkor, that microbial pathogenicity is enhanced considerably.

In marked contrast, hen egg albumen *in vitro* at 39.5°C did not allow extensive multiplication of *Escherichia coli* C3650 until the ovotransferrin was completely saturated with Fe^{3+} (Table 11). At levels below 100%, death of the bacteria occurred. The failure to satisfy completely the chelating potential of ovotransferrin may have been a major error in those early studies that failed to produce growth of micro-organisms in egg albumen deemed to have been saturated with iron. I have assumed that ovotransferrin and serum transferrin share a common mechanism in their reactions with Fe^{3+} complexes involving an exchange of ligands (Bates and Schlabach, 1973). The ligands in the co-ordination sphere of the Fe^{3+} complex are gradually replaced by the binding groups of the protein until the iron is totally co-ordinated by the protein.

The reaction of serum transferrin with complexes like ferric nitrilotriacetate and ferric citrate occur quite readily (Bates and Wernicke, 1971 ; Bates *et al.*, 1967); however ferric salts such as $FeCl_3$, that form inert spherical polymers, are not readily attacked by the transferrins. The major reason why the ligands forming a stable cross-linked iron nucleus are not displaced by the binding groups of the protein is probably one of steric hindrance. Thus when ovotransferrin was titrated with $FeCl_3$ at high pH there was no clear end point (Fig. 19). When compared with ferric salts, ferrous complexes gave a linear

response and a clear saturation point when titrated against ovotransferrin (Fig. 19). This is because ferrous salts exist as low molecular weight complexes with readily dissociable ligands.

Although Garibaldi (1960) observed growth of Pseudomonas fluorescens and an Aeromonas sp. in egg white which was less than 100% saturated with iron, his work was done at an incubation temperature of 28°C much removed from the incubation temperature used throughout most of this study.

In experiments with serum in vitro three effects can occur ; some bacteria grow normally (Bullen and Rogers, 1969) ; some are killed rapidly (Fletcher, 1971) ; with others there is a short period of growth followed by prolonged bacteriostasis (Bullen and Rogers, 1969). The presence of complement in serum has been deemed to be responsible for this bactericidal activity. It has long been known that this material together with specific antibody can cause lysis of sensitive strains of Escherichia coli. In support of this, Bullen and Rogers (1969) showed that complete destruction of the complement by heating changed the bactericidal property of rabbit serum to a bacteriostatic one. Both of these effects could be overcome by saturation of the transferrin with Fe^{3+} or by the addition of haem compounds (Bullen and Rogers, 1969) which are not bound by transferrins.

Human milk also has a powerful bacteriostatic effect on bacteria (Bullen et al., 1972). It contains large quantities of lactoferrin (2-6 mg/ml) but relatively small quantities (10-15 µg/ml) of transferrin (Masson and Heremans, 1971). Although lactoferrin itself had some inhibitory effect, the combination of this with specific antibody had a more powerful effect resembling that of milk itself (Bullen et al., 1972). This effect was also demonstrated with serum transferrin and specific antibody (Rogers, 1973). In both cases the bacteriostatic

properties of the iron-binding proteins and antibody were abolished by the addition of iron sufficient to saturate the transferrins.

In the absence of an adequate immune response and the localized nature of the protection afforded by the maternal antibody (Yamomoto and Bigland, 1966), the developing embryo must depend on non-specific defence mechanisms. Hen egg albumen has a strong bactericidal effect on bacteria at normal incubation temperatures and pH values. This effect is greater on Gram-positive bacteria (Figs. 23-26) than Gram-negative ones (Figs. 27-31) and only the latter are able to grow when ovotransferrin is saturated with iron. Bacteriostasis can be induced by lowering the incubation temperature of egg white to 30°C or below (Figs. 42 and 44) or by reducing the pH to values below pH8 (Figs. 45 and 46). In both of these cases some bacteria were able to grow very slowly after a day or two. Thus the bactericidal property of hen egg albumen may be attributed to the presence of an iron-binding protein, ovotransferrin, together with a high incubation temperature and/or pH, and probably small amounts of readily available nitrogenous compounds (Fig. 22).

The bacteria that can grow in serum must possess a specific mechanism for acquiring iron from partly saturated transferrin. Apart from direct proteolysis of the iron-protein complex, for which there is no evidence, there are only two ways by which this could be achieved. One is the direct interaction between the bacterial cell envelope and the transferrin molecule in a reaction analagous to that of transferrin and the reticulocyte (Fletcher and Huehns, 1968). This may well happen with highly virulent organisms such as Pasteurella septica (Bullen et al., 1968). The other and most probable mechanism is the microbial secretion of a low molecular weight iron chelator capable of removing iron from the transferrin molecule and re-entering the cell where it

gives up its iron.

Rogers (1973), working with two serotypes of Escherichia coli (0111 and 0141), showed that there was a direct relationship between the ability to synthesize catechols and the virulence of these organisms for mice. The 0111 strain, which was sensitive to complement, had an LD_{50} of 1×10^8 for mice. It was killed by normal rabbit serum and inhibited by heated serum. The complement-resistant strain 0141 had an LD_{50} of 3×10^6 for mice and showed inoculum-dependent growth in fresh serum - small inocula failed to initiate growth whereas large ones did. Rogers (1973) suggested that the large inoculum might be producing an essential metabolite that was enabling the bacteria to grow. Culture fluids of Escherichia coli 0141 grown in the presence of a synthetic iron-chelator, ethylene diamine-di-o-hydroxyphenyl acetic acid, E.D.D.A., contained derivatives of 2,3-dihydroxybenzoic acid which were capable of abolishing the bacteriostatic effects of the serum. One of these compounds, tentatively identified with enterobactin, was capable of removing iron from its complex with transferrin. When Escherichia coli 0111 was also found to produce catechols in low-iron media the question arose as to why this organism failed to multiply in serum. This was explained by the presence of antibody in the serum which inactivated the synthesis of enterobactin (Rogers, 1973). Further work showed that these iron-sequestering substances could be described as "virulence factors" since organisms lacking them would be unable to multiply within the host. This was shown in vivo when the compound isolated from strain 0141 produced a fatal infection when administered with an otherwise harmless dose of Escherichia coli 0111 into mice (Rogers, 1973).

Mycobactins, the iron-sequestering molecules formed by the Mycobacteria (Snow, 1970), may also play an important role in infection

as they can also remove iron from transferrin thus neutralizing serum tuberculostasis (Kochan et al., 1971).

Results similar to those obtained in serum have also been obtained in human milk and bovine colostrum, both of which exert a bacteriostatic effect on Escherichia coli 0111 and both of which can be abolished by saturating the iron-binding potential of lactoferrin with Fe^{3+} (Bullen et al., 1972 ; Reiter et al., 1975). This effect depends however on the addition of bicarbonate to counteract the iron-mobilizing effects of the large amounts of citrate normally present in these secretions (Reiter et al., 1975). Not all strains of Escherichia coli are inhibited by human milk and bovine colostrum (Griffiths and Humphreys, 1976), a feature that can be attributed to lack of specific antibody.

The two most commonly studied serotypes of Escherichia coli (0111 and 0141) used in work on the antibacterial systems in mammalian milk and serum, were included in this study in order to achieve as close a comparison as possible between egg white and the above two. In this respect they did not differ from any other Gram-negative bacteria in egg albumen at 39.5°C. They were killed in egg white at pH 9.2 while their populations remained static at pH 7.6. In contrast to serum and mammalian milk, which have a pH around neutral, inoculum dependent growth of Escherichia coli 0141 could not be demonstrated in hen egg white at either of the above pH values. It has been suggested that 2,3-dihydroxybenzoic acid and similar compounds which can reverse the bacteriostatic effect of ovotransferrin on Salmonella typhimurium, may contribute to the spoilage of eggs by facilitating bacterial multiplication in the white (Garibaldi, 1970). If the production or presence of iron transport compounds is a prerequisite for an organism to establish itself, it is vital that the egg possesses some means of blocking siderophore synthesis or inactivating any siderophores produced

by the invading organism.

One mechanism of doing this has already been discussed - the suppression of siderophore synthesis (or release) by antibody in serum (Rogers, 1973). The same mechanism may also apply to mammalian milk. Garibaldi (1971, 1972) has shown for Salmonella typhimurium and a fluorescent pseudomonad, and Kochan (1977) for Escherichia coli that the ability to produce iron transport compounds is substantially reduced by a small increase above the optimum temperature required for growth. As a result it has been suggested that the reduction in mammalian serum iron, coupled with an elevation in body temperature (fever), is a co-ordinated host defence mechanism against invading pathogens (Weinberg, 1974 ; Kluger and Rothenburg, 1979).

Although the production of extracellular catechol-like compounds accompanying growth was demonstrated (Fig. 51) in de-ironised media at 39.5°C (pH 7.6), they were not detected in hen egg albumen (pH 9.2) at the same temperature. This suggests that alkaline pH values rather than elevated temperatures were responsible for the inhibition of siderophore production/release in hen egg albumen. Enterobactin is an extremely unstable molecule ; its half-life in aqueous solution (pH 7) is of the order of hours. It suffers both oxidation of the ring hydroxyl groups and hydrolysis of the lactone. The hydrolytic products of enterobactin are also iron chelators but the stability of the complexes they form decreases with the decreasing complexity of the ligand (O'Brien et al., 1971). If enterobactin was produced in hen egg white (pH 9.2), non-enzymatic hydrolysis of the type described above would probably occur (plates 17 and 18) breaking the trimer down into simpler monomer constituents incapable of scavenging iron to the same extent as enterobactin (Hancock et al., 1977). Further indication of the ineffectiveness of these lower constituents to obtain iron from an

iron-binding protein was given when a commercial preparation of 2,3-dihydroxybenzoic acid failed to stimulate growth of Escherichia coli C3650 in hen egg albumen (Fig. 55). Indeed the complete absence of catechol-like compounds in egg white at this pH tends to suggest that no iron transport compounds of any type were produced. In addition, although the production of these compounds was not investigated at the lower pH, the fact that unless purified enterobactin was added (Fig. 53), the growth of Escherichia coli 0141 was inhibited in egg white even after 24h implies that production of siderophores was completely suppressed at both pH values. It would appear then that whereas serum and probably milk rely on an iron-binding protein and antibody to prevent micro-organisms from obtaining iron, interplay of an alkaline pH and ovotransferrin would seem to be of major importance in eggs.

It is not known if other bacterial chelates are involved in the microbial infection in eggs. Indeed in this study no attempt was made to investigate the ability of micro-organisms to secrete and re-absorb hydroxamate siderophores in egg albumen. Hydroxamate siderophores are more commonly found in fungi such as Penicillium, Aspergillus and Fusarium. Only a few bacteria are known to secrete hydroxamate siderophores during iron deprivation : Bacillus megaterium (Schizokinen) ; Enterobacter aerogenes strain 62-1 (Aerobactin) ; Arthrobacter pascens (Arthrobactin or terregens factor) and some species of Pseudomonas. In the case of members of the last mentioned genus, pyoverdine$_{pf}$, the yellow-green fluorescent pigment of Pseudomonas fluorescens (Meyer and Abdallah, 1978a), is the siderophore. Similar iron sequestering pigments have also been observed in Pseudomonas aeruginosa, Pseudomonas chloraphis and Pseudomonas putida (Meyer and Abdallah, 1978b). These hydroxamates should not be confused with pyochelin, a phenolate siderophore isolated from Pseudomonas aeruginosa (Cox and Graham, 1979). Although pyoverdine$_{pf}$

has been shown to be chemically unstable under mildly alkaline conditions (Meyer and Abdallah, 1978a), very little is known about the stability of other bacterial hydroxamates. In fact the enhanced pigment production by pseudomonads in the presence of ovotransferrin or egg white observed by Feeney and Nagy (1952) and Garibaldi (1967) was due to the secretion of iron-binding hydroxamate siderophores in response to the low concentration of free iron. Indeed these fluorescent pigments can often be seen in egg whites that have been contaminated during some stage in modern egg processing plants (Board, personal communication). In addition the fluorescent pigments produced by many of the contaminants found in the waterfowl egg whites (Table 22) may be further evidence to suggest that bacteria with the ability to produce hydroxamate siderophores are better suited to survive the high pH obtained by egg albumen.

In the past workers have tended to describe the antimicrobial defence system of albumen by merely listing its principal protein components. From the results of this study it has become apparent that the bactericidal activity of avian albumen depends not only on these protein constituents but also on the high alkalinity of the albumen. For, in addition to the influence pH has on the binding of iron by ovotransferrin (iron is bound more tightly at high pH), the high alkalinity may possibly prevent micro-organisms from scavenging this element because of the inhibition of siderophore production (Fig. 52) and uptake (Fig. 54). The incubation temperature of the hen's egg may also play some role in the inhibition of micro-organisms ; the results in this study suggest this but a full understanding of this will have to wait further studies. Perry and Weinberg (1973) attempted to explain the differences in the survival of bacteria at different temperatures in iron deficient media by postulating a decreased iron requirement at

lower temperatures due to lower metabolism. Whilst this would be true at temperatures below 20°C (Fig. 50) it would be unlikely to occur at higher temperatures such as 25 or 30°C. One possible explanation may be that bacteria have one less restraint to overcome when suspended in unsupplemented hen egg white at pH 9 or above and temperatures below the incubation temperature of the hen.

This last statement is also probably true for yeast vegetative cells in hen egg albumen. An increase in incubation temperature from 30 to 39.5°C or pH from 7 to 9 resulted generally in a greater extent of decline of the yeast populations (Figs. 36-39). In marked contrast to bacteria however, the availability of free iron did not appear to be the major factor of yeast inhibition in hen egg albumen. Thus with the exception of the brewing yeast Saccharomyces cerevisiae (Fig. 38), iron saturation of ovotransferrin did not allow extensive growth of these organisms to occur (Figs. 36-39). Although hydroxamate siderophores, based on the compound rhodotorulic acid, have been demonstrated in cultures of wild yeasts such as Sporobolomyces and Rhodotorula spp. (Atkin et al., 1970) no such molecules have been observed with Saccharomyces cereviseae (Atkin et al., 1970). The apparent independence upon iron exhibited by the yeasts in this study was an unexpected observation. One might expect that these micro-organisms whose nutritional requirements are generally more complex than most bacteria associated with addled eggs, would be even more susceptible to iron deprivation than bacteria. The inhibition of yeasts may have been due to avidin, another chelating agent present in avian egg whites. Although this protein is present in hen egg white at a much lower concentration (0.1% of the total protein) compared with ovotransferrin (13.7% of the total protein), its strong affinity for the vitamin biotin has provided the basis for assaying for biotin-requiring micro-organisms

(Wright and Skeggs, 1944) including the yeasts (Hertz, 1943).

The importance the interplay of high alkalinity and iron-deficient conditions was emphasized by observations of the inhibition of vegetative cell outgrowth from bacterial endospores suspended in hen egg albumen (Tranter and Board, 1982). Of the three main stages in Bacillus cereus T spore development - germination ; swelling ; outgrowth (Hitchins et al., 1963) - only the last two stages were affected by hen egg white. The fact that germination was not affected (Fig. 34) is a feature in common with a lot of other food preservatives such as nisin, sorbate and nitrite (Gould, 1964), and is in keeping with the concept of germination being an irreversible process which, when compared with vegetative cells, is relatively insensitive to its environment.

Takagi et al. (1960) showed that the spore cortex but not the spore coat was disrupted early in spore germination processes thus enabling the spore core to swell (Hitchins et al., 1963) during rehydration. The second phase of swelling that occurred with spores suspended in hen egg white (pH 9) supplemented with L-alanine and inosine (Fig. 2 ; Tranter and Board, 1982) resulted in some cases in a barely visible spore cortex that was squeezed up against the spore coats (plates 7a and b). It was notable that a marked swelling has also been observed when outgrowth was inhibited by other chelating agents such as ethyl picolinate (Pandey and Solanki, 1980) and polyphosphates (Gould, 1964).

The inhibition of outgrowth from bacterial endospores by an iron chelating protein was shown by Ashton and Busta (1967) and Oram and Reiter (1968), who studied spores of Bacillus stearothermophilus in media containing lactoferrin at pH 7.0. The inhibition was relieved by iron. This element overcame the inhibition of outgrowth from spores of Bacillus cereus T in egg white but only when the pH was lowered from 9.0 to 7.9 (Tranter and Board, 1982). About 2% of spores in unsupplemented

egg white (pH 9.0) attempted outgrowth but the resulting "cells" appeared to be weak structurally and they never became free of the spore coat (plate 9d). Electron microscopic examination of such "cells" showed that the cellular integrity was affected and lysis of the cell envelope appeared to be occurring (plates 16a and b). A possible explanation for this may be that the spores, derived from lysozyme-resistant mother cells, were producing vegetative cells that were susceptible to the lysozyme present in hen egg white. Thus they may have been behaving in a similar way to the delay of outgrowth, by purified hen egg white lysozyme, from spores derived from lysozyme-resistant mother cells of Clostridium tyrobutyricum (Wasserman and Teuber, 1979) which also exhibited delayed outgrowth when purified lysozyme from hen's egg white was present in the medium.

The vegetative cells that emerged from Bacillus cereus T spores in iron-supplemented hen egg albumen (pH 7.9) had a similar morphology to the normal cells that had emerged from spores in a rich medium but they gave a granular Gram-reaction (plate 12) due to the presence of large amounts of lipid (plate 13). Although these lipid granules, probably poly-β-hydroxybutyrate (PHB), are common in the cytoplasm of many Bacillus spp., they tend to occur as a storage product largely in physiologically old cells and are generally few in number during active growth of the micro-organism. This suggests that the emerging cells are not able to achieve normal growth immediately after emergence. The amount of lipid material decreased when the cells were left overnight and many of the cells formed refractile spores (plate 14). In Bacillus megaterium the sporulation process depends on the rate of utilization of PHB which serves as an endogenous carbon and energy source (Slepecky and Law, 1961).

There was some outgrowth from spores in unsupplemented hen egg

white (pH 7.9) but these "cells" were of a bizarre form and appeared to have had problems with cell division (plate 11). These "cells" had only small amounts of lipid and did not form spores. This suggests that iron is a possible requirement for sporulation and perhaps it is involved in PAB utilization also.

Physiological implications of iron deprivation and reasons for death

As iron is an essential nutrient, its occurrence at very low levels has far reaching consequences on microbial structure and function. Catalase activity is markedly reduced in many microbes subjected to iron limitation (Waring and Werkman, 1944 ; Theodore and Schade, 1965). A similar reduction occurs in the cytochrome content of iron-deficient micro-organisms (Waring and Werkman, 1943, 1944). These workers showed that when iron was added to depleted media, the requirements of the cytochromes were satisfied before those of the iron dependent enzyme systems. Iron-deficient cells of 'Staphylococcus pyogenes' fail to oxidise all the glucose and lactate to acetate and acetoin (Theodore and Schade, 1965) and 'Aerobacter cloacae' ferments glucose to formate and lactate with little CO_2 and H_2 due to a deficiency of various enzymes including formic dehydrogenase, formic hydrogen lyase and hydrogenase (Waring and Werkman, 1942). The deoxyribonucleic acid (DNA) content of Mycobacterium smegmatis falls markedly when the iron supply in a medium is limited probably as a result of some impairment of the ribonucleotide reductase system (Coughlan, 1971).

The decrease in the amount of the synthesis of the cytochrome and iron-containing proteins, many of which are intensely coloured, during iron limitation contributes to changes in microbial appearance. Cells of Serratia marcescens in unsupplemented hen egg albumen (Fig. 49) gradually lost their pigment whereas cells of the same organism retained their pigment-producing potential in iron supplemented hen egg white.

This phenomenon was first noted by Bunting (1940) but it was Williams et al., (1956) who showed that iron was actually incorporated into the red pigment, prodigiosin.

In many cases the availability of iron has been found to affect the size, shape and growth characteristics of micro-organisms. Indeed iron-deficient cells of Mycobacterium smegmatis (Winder and O'Hara, 1962) and Escherichia coli (Ratledge and Winder, 1964) occurs as filaments.

Considerable attention has been paid to the bacteriostatic effect of antibody and iron-binding proteins on Escherichia coli. The speed at which inhibition of growth by serum occurs suggests that progressive iron limitation within the cell is not of primary importance. Indeed Griffiths (1971) has envisaged a rapid depletion of iron from a specific and crucial cellular role. He showed that specific antibody and transferrin inhibited Pasteurella septica by interfering with the biochemical metabolism of the cell. Thus net ribonucleic acid (RNA) synthesis began to decline 15min after the addition of antiserum ; this was followed by inhibition of net protein synthesis and finally DNA synthesis. Similar changes in macromolecular synthesis have been observed in Escherichia coli O111 exposed to antiserum (Melching and Vas, 1971). The addition of iron, while not preventing the initial inhibition, allowed net macromolecular synthesis and cell multiplication to be resumed in the same sequence as its initial inhibition.

One result of these observations was the appearance of abnormal transfer ribonucleic acid (tRNA) species in bacteria (Rosenberg and Gefter, 1969). The chromatographic profile of several tRNA's from iron-deficient Escherichia coli differed markedly from those of iron-sufficient cells. Tyrosyl-tRNA and phenylalanyl-tRNA from iron-deficient cells appeared earlier in the chromatographic elution profile. Griffiths (1972) found that Escherichia coli O111 inhibited by horse serum contained

abnormal phenylalanyl-tRNA. Addition of iron to saturate the transferrin prevented the formation of abnormal tRNA. Similarly addition of hematin resulted in the conversion of the abnormal to the normal form. Iron may be required for the synthesis of the 2-methylthio group on the isopentyl adenosine residue adjacent to the anticodon region in tRNA without which the molecule can not function normally (Rosenberg and Gefter, 1969). Griffiths (1972) suggested that it was the complete absence of normal phenylalanyl tRNA from bacteria inhibited by serum that was responsible for bacteriostasis.

In contrast to the observations made in serum, the growth of bacteria in an iron-deficient medium is usually only slowed down and slow growth may persist for upwards of 24h or longer (Ratledge and Winder, 1964). In an attempt to mimic the effects of hen egg albumen, Escherichia coli 0141 was inoculated into iron-deficient medium buffered between pH 7.0-9.0 (Figs. 47 and 48). This resulted in a reduction of growth only. Wettstein and Stent (1968) showed that Escherichia coli grown in an iron-depleted medium contained both abnormal and normal $tRNA^{phe}$. This suggests that unlike the situation in serum, enough of the normal component was still present to allow protein synthesis to occur almost unperturbed.

Bactericidal Systems in Mammalian Serum, Milk and Avian Egg White

Although the antimicrobial nature of the three systems noted above became known at about the same time - at the turn of the century - the systems in milk and egg white have only attracted intermittent attention. It is only over the past 15 years that the milk system has received sustained study (Reiter, 1976) ; the system in egg white still awaits clarification. The bacteriostatic mechanism of these three systems has already been discussed, namely their dependence on the commonly occurring iron-binding proteins together with some other component, antibody in

the case of human sera and milk, which prevents the production and/or uptake of iron chelating agents. The non-cellular bactericidal mechanisms in mammalian serum, milk and avian egg white appear to differ in that each one has evolved a particular method for inhibiting micro-organisms.

In the late nineteenth century it was recognised that the bactericidal activity of fresh serum depended on two factors. The first, heat stable and specific for each organism, was identified as "antibody" and later shown to belong to the immunoglobulins. The second factor, heat labile and non-specific, was designated "complement". At present, more than 20 components have been shown to interact in the complement system (Adinolfi, 1981). The interaction of the complement system with antigen-antibody complexes or directly with bacterial polysaccharides results in the sequential activation of the various components and the formation of multimolecular structures which cause bacterial cell lysis. Although some strains of Escherichia coli are insensitive to this material others are not ; furthermore mouse serum complement does not appear to be bactericidal (Marcus et al., 1954). This anomaly of complement behaviour suggests that the factors controlling bacterial growth in vivo are still not fully understood.

Mammalian milks differ significantly from each other. For example, human milk contains more lactose but less protein and phosphate than bovine milk and also has a poorer buffering capacity than the other (Bullen and Willis, 1971). Human milk has a powerful bacteriostatic effect on Escherichia coli (Bullen et al., 1972) but bicarbonate has to be added before this can be expressed. The non-specific bactericidal effects on bacteria observed with bovine milk (Reiter et al., 1976) are due mainly to the presence of a lactoperoxidase system together with other lesser studied basic antibacterial proteins such as properdin,

conglutin and the vitamin binders for B_{12} and folate (Reiter, 1981).

Although lactoperoxidase occurs in the milk of many species its level in human milk has been estimated at about 5% of the concentration in bovine milk. The system is less important in human milk because of the low amounts of hydrogen peroxide and thiocyanate which are needed for the system to function. Thus human milk becomes bactericidal only after the addition of a source of H_2O_2 and SCN^- (Reiter, 1981).

The bactericidal system in avian egg white is not restricted to birds of the domesticated variety. This antibacterial action was also found in the albumen of eggs of various waterfowl species (Figs. 56 and 57). The system in egg albumen seems to operate in the absence of any antibody-complement system and an enzyme system (apart from possibly lysozyme). It depends on the high alkalinity, achieved shortly after lay by the loss of CO_2 across the shell, super-imposed upon an iron-depleted, nutrient deficient environment. From the results in this study the incubation temperature of the hen may also play a part in preventing microbial growth.

Although naturally occurring alkalinophilic micro-organisms are found in soils, alkaline lakes and springs (Grant and Tindall, 1980) where the pH can be as high as 10-12, the optimum pH for the growth of most micro-organisms is normally around neutrality. Despite this some organisms including the enteric bacteria are tolerant to pH values near 9-10. Indeed the ability to grow in media of about pH 10 is one of the characteristics of the enterococcus group (Chesboro and Evans, 1959). The fact that many micro-organisms are able to grow at extreme pH values suggests that they have some mechanism for maintaining their internal pH close to neutrality. The cytoplasm contains many alkali-labile and acid-labile molecules such as adenosine triphosphate (ATP) and DNA which would be dramatically affected if the

intracellular pH was always the same as the extracellular pH. Acidophiles which exclude H^+ (Brown et al., 1980) and alkalinophiles which exclude OH^- or retain H^+ (Langworthy, 1978) both maintain their intracellular pH values close to normal physiological values. The complete exclusion of ions which is thought to occur by metabolically active pumping mechanisms (Langworthy, 1978), would very likely place a stringent demand on cellular energy. In summary the role of alkaline pH in the defence system of egg white may be two-fold. Firstly, it imposes additional stress upon an iron-deficient and hence energetically insufficient cell. Secondly, it appears to prevent the bacteria from using their usual scavenging processes when the medium is depleted of extracellular iron.

In discussions of avian embryology there is a distinct tendency to ignore those systems which, through protecting the white and yolk from microbial infection, permit embryo development in an environment isolated from that of the parents. The work done in this study has shown that an antimicrobial system exists in avian egg whites which can operate in the absence of any neural or hormonal control and without the need for energy consumption. Indeed the uniqueness of this system may represent an extremely old form of defence which has provided the basis for the development of the immunodefence systems observed in higher organisms.

POSSIBLE PRACTICAL IMPLICATIONS OF THE ANTIMICROBIAL SYSTEM OBSERVED IN AVIAN EGG WHITES

It is now generally recognized that colostrum and milk contain a multifactorial antibacterial system that protects the newborn until the body's own defence system is developed sufficiently. A noteworthy change in infant nutrition in this century has been the move towards breast feeding and away from the use of heat-treated cows milk. Robinson (1951) reported that the morbidity and mortality rates among 3,266 children in Great Britain, were lowest in breast-fed infants, highest in bottle-fed infants and intermediate in those partly breast and partly bottle-fed. More recently Mata et al. (1976) produced convincing evidence from a survey of mothers in Guatamala that enteric infections were lowest in suckled infants despite appalling conditions of hygiene.

Recent evidence suggests that there are three possible defence mechanisms operating in the breast-fed infant. Firstly, there is the direct inhibition of growth of Escherichia coli in the small intestine (Bullen et al., 1972). For milk to be effective, it must be relatively unchanged when it reaches the small intestine. Indeed Mason (1962) showed that little or no proteolysis occurred in the stomach of the suckling infant, presumably as a consequence of the trypsin inhibitor found in human milk (Laskowski and Laskowski, 1951). Bullen et al., 1972 also demonstrated a rapid decline in the numbers of orally administered Escherichia coli 0111 in both the small and large intestine of suckling guinea pigs which could be reversed by the addition of iron indicating that the protein lactoferrin was reaching these organs unaltered. Secondly, there is the possibility that milk could prevent adhesion of pathogenic strains of Escherichia coli to the gut wall (Nagy et al., 1976). Thirdly, human milk is known to encourage the

growth of a bacterial flora which tends to exclude Escherichia coli and other enteric bacteria from the large intestine (Bullen and Willis, 1971). Human milk with its high lactose content and low buffering capacity appears to provide ideal conditions for the development of bifidobacteria. These organisms dominate the gut flora and produce large amounts of acetate which lowers the pH to 5.0-5.5 thereby suppressing the growth of enteric bacteria (Bullen, 1981). In contrast, bottle-fed infants receive a high protein, high phosphorous, low lactose diet. No acetic acid is produced by bacterial fermentation and the pH remains relatively high at 6.5-7.0 in a well buffered medium. This discourages organisms such as Bifidobacterium and encourages a putrefactive flora (Bullen and Willis, 1971).

For various reasons it is not always possible to breast-feed infants ; the baby may be premature or the mother may be unable to produce sufficient milk. While artificial feeding of the newborn is relatively successful in a sophisticated society such as our own because of the high standard of hygiene, it can be a dangerous practice in underdeveloped countries. The production of milk formulations for infants involves heating bovine milk and processing it to a dried powder which destroys the inhibitors present. The preparation of such milk formulations together with infant weaning foods (Rowland and McCollum, 1977 ; Barrell and Rowland, 1980) almost invariably involves water obtained from village wells which can be contaminated to the degree of 10^{10} bacteria per millilitre (Rowland and McCollum, 1977). A prepared food may then be stored without being cooked for as long as 12h before consumption. It is little wonder therefore that such products are unsafe especially to the young infant. Similarly, feeding calves on powdered milk offers little protection against infection as the antimicrobial factors have been destroyed by the heat processing (Ford et al., 1977).

This is well illustrated by the difficulty in rearing calves on formula feeds based on dried milk unless the hygienic conditions are so good that rearing becomes uneconomical.

The microbial content of spray-dried baby milk reconstituted with sterile deionised water or sterile buffer is shown in Table 24. The addition of hen egg albumen to this reconstituted milk (1:5) had two major effects. Firstly the iron content of the milk was chelated by the albumen's ovotransferrin and secondly the pH of the milk rose from 7.0 to 8.5. Moreover the addition of egg white restricted microbial growth in the milk, an effect that was accentuated by an increase in incubation temperature (Table 25). The results in Table 26 indicate that this effect was not purely an effect of increased pH as milk reconstituted in phosphate buffer (pH 8.0) still had a high microbial content when incubated overnight compared to milk with egg white at the same pH (Table 26 ; plate 19). The addition of iron to a mixture of milk and egg white poised at pH 6.0 had an opposite effect in that it reduced the microbial content. This may have been due to a decrease in affinity for the iron by ovotransferrin at this pH ; the reduction in numbers arising from a toxic excess of this element.

The effects of egg albumen on the microflora of dried milk can be mimicked (Table 27) to some extent by the addition of a commercial preparation of ovotransferrin, the principal inhibitory component in egg white. Although the reduction in numbers was not quite so marked as with egg white itself, ovotransferrin did achieve some reduction in the growth of micro-organisms during incubation. It was notable during the work with ovotransferrin in milk that the addition of the chelate selected for a microflora composed almost entirely of Bacillus spp. where before a mixed population containing members of the genera Bacillus, Micrococcus, Pseudomonas and coryneform organisms were present.

Although the major part of this study was concerned with the identification and inter-relationship of the factors involved in the antimicrobial defence system of avian egg albumen, an integral part of the work was to examine any practical application the natural system might have with respect to prolonging the shelf life or preventing the contamination of commercially available foodstuffs. The search for inhibitors of microbial growth that could be used as effective preservatives of perishable foodstuff and feedstuff is particularly active at the present time because of the strong lobby against the use of 'chemicals' and preservatives in food processing. This in turn is forcing the manufacturer to turn his attention away from synthetic and relatively unknown preservative systems towards more naturally occurring and therefore commercially acceptable systems.

It is becoming an increasingly popular idea to supplement formulated feeds, like the ones described above, with one or more components of the antimicrobial defence systems discussed in this thesis. Indeed Reiter (1981) has discussed the idea of supplementing powdered milk with the lactoperoxidase system to suppress the bacterial contamination that occurs during its reconstitution. He also states that hen egg white lysozyme has been added to baby feeds but with little success. Although serum, milk and egg white all contain this enzyme, the _in vivo_ or _in ovo_ role of this enzyme is still unknown. It is unfortunate that because egg white is the richest and most easily available source of lysozyme, most of its anti-bacterial activity has been incorrectly matched with lysozyme from either of the other systems (Reiter, 1978). It is little known for instance, that hen egg white lysozyme has a much lower activity than lysozyme from either of the other systems. Reiter's observations confirm the contention upheld by this study that lysozyme does not play a primary role in the antibacterial activity of hen egg white. The addition of unsupplemented hen egg white (Table 26) or iron-

free purified ovotransferrin (Table 27) to powdered baby milk however suggests that these are better suited than lysozyme to deal with bacterial contamination. Indeed the anti-protease factors (ovomucoid and ovoinhibitor) in egg white could well play an important role in ensuring that the antimicrobial proteins are protected in the upper portion of the infants' gastro-intestinal tract.

TABLE 24

THE MICROBIAL CONTENT OF RECONSTITUTED DRIED BABY MILK.

Micro-organism	Media	Range of viable content
Total Aerobic count	Plate Count Agar *	1-1500
Aerobic spore formers	P.E.G.Y.E. Agar *	0-100
Lactobacilli	M.R.S. Agar *	-
Moulds	Malt Agar *	0-3

* Stored at 30°C and 37°C and examined daily over a three day period.

Number of samples examined = 12

TABLE 25

THE EFFECT OF EGG WHITE AND TEMPERATURE ON THE GROWTH OF THE MICROFLORA OF RECONSTITUTED DRIED BABY MILK.

Incubation Temperature (°C)	Viable counts / ml *	
	Reconstituted Milk for 18h	Reconstituted Milk + Egg White incubated for 18h
6	8.1×10^2	15
30	4.4×10^2	5
39.5	2.2×10^7	-

Initial Microbial load = 10 viable cells/ml

* Plate count agar at 30°C for 3 days and examined daily.

TABLE 26

THE EFFECT OF pH AND IRON CONCENTRATION ON THE INHIBITION OF THE MICROFLORA OF RECONSTITUTED BABY MILK BY HEN EGG WHITE.

	Viable count / ml *		
pH	milk held at 37°C for 18h	milk containing egg white held at 37°C for 18h	milk containing iron-saturated egg white held at 37°C for 18h
6.0	6.62×10^7	7.73×10^6	1.5×10^2
7.0	5.55×10^8	25	3.72×10^{10}
8.0	10^8	4.00×10^3	3.20×10^8

Initial Microbial load = 2.5-3.5 viable cells / ml

* Plate count agar incubated at 30°C and 37°C for 3 days and examined daily.

TABLE 27

THE EFFECT OF OVOTRANSFERRIN AND pH ON THE GROWTH OF THE MICROFLORA OF RECONSTITUTED DRIED BABY MILK.

	Viable count / ml *		
pH	milk held at 37°C for 18h	milk containing egg white held at 37°C for 18h	milk containing ovotransferrin held at 37°C for 18h
7.2	2.24×10^9	2.00×10^2	1.5×10^3
8.6	3.46×10^4	-	2.5×10^3

Initial Microbial load = 2.0-2.5 viable cells/ml

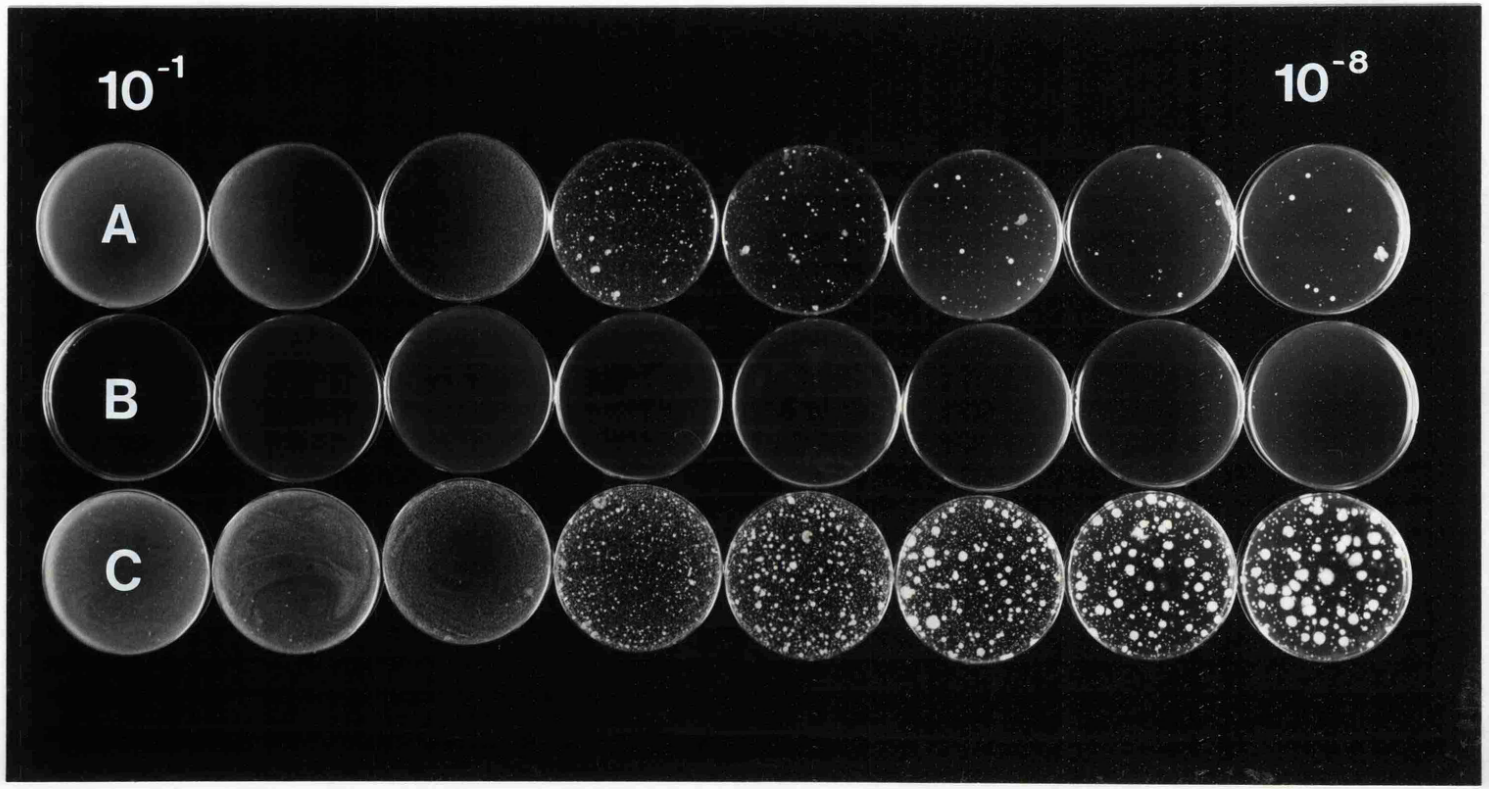

Plate 19. The effect of adding egg white to reconstituted baby milk on the growth of the bacterial flora of the milk (see text). A, milk; B, milk containing egg white and C, milk containing egg white and iron.

APPENDIX

LIST OF GENERIC AND SPECIFIC NAMES

The avian species in this thesis are referred to by their common names; generic and species names are listed below.

Cassowary	*Casuarius aruensis*
Duck, Black-headed	*Heteronetta atricapilla* *
Duck, Comb	*Sarkidiornis melanotos melanotos* *
Duck, Khaki Campbell	Domestic
Duck, Mandarin	*Aix galericulata*
Duck, North American Ruddy	*Oxyura jamaicensis*
Duck, Peking	*Anas platyrhynchos*
Duck, South American Ruddy	*Oxyura* sp.
Duck, White-faced Whistling	*Dendrocygna viduata* *
Duck, White-winged wood	*Cairina moschata*
Fowl, domestic	*Gallus domesticus*
Goldeneye, Barrow's	*Bucephala islandica*
Goldeneye, European	*Bucephala clangula clangula*
Goose, Aleutian Canada	*Branta canadensis*
Goose, Barnacle	*Branta leucopsis*
Goose, Emperor	*Anser canagicus*
Goose, Greater Snow	*Anser caerulescens atlanticus* *
Goose, Pacific White-fronted	*Anser albifrons*
Goose, Red-breasted	*Branta ruficolis* *
Goose, White Embden	Domestic
Penguin, Adelie	*Pygoscelis adeliae*
Pheasant, Golden	*Chrysolophus pictus*

Quail, Japanese	*Coturnix coturnix japonica*
Shelduck, European	*Tadorna tadorna*
Smew	*Mergus albellus*
Spotbill, Chinese	*Ansa paecilorhyncha zonorhyncha* *
Swan, Bewick	*Cygnus columbianus*
Swan, Black	*Cygnus atratus*
Swan, Black-necked	*Cygnus melanocoryphus*
Teal, Puna	*Anas versicolor puna*
Teal, Ringed	*Calonetta leucophrys*

* Authority

"A coloured key to the Wildfowl of the World" (1972)

Peter Scott,
The Wildfowl Trust : Cambridge, Glos.

APPENDIX

COMMON METHODS FOR THE PURIFICATION OF AVIAN EGG WHITE PROTEINS INVOLVED IN THE ANTIMICROBIAL DEFENCE SYSTEM.

Lysozyme

Direct crystallization from egg white containing 5% (w/v) NaCl at pH9.5	Alderton and Fevold (1946)
Ion exchange chromatography on carboxymethylcellulose (CMC)	Rhodes et al. (1958)
Ion exchange chromatography on dietylaminoethanolcellulose (DEAE)	Mandeles (1960)
Ion exchange chromatography followed by gel filtration	Jollès et al. (1962) Arnheim et al. (1969)

Ovotransferrin

$(NH_4)_2SO_4$ fractionation from egg white	Osborne and Campbell (1900)
Ethanol fractionation from egg white	Bain and Deutsch (1948)
Ion exchange chromatography on carboxymethylcellulose	Rhodes et al. (1958)
Crystallization from egg white using $(NH_4)_2SO_4$ followed by ion exchange chromatography	Azari and Baugh (1967)

Avidin

Acetone and salt fractionation from egg white	Eakin et al. (1941)
Absorption onto bentonite	Fraenkel-Conrat et al. (1952a)
Ion exchange chromatography on CMC	Rhodes et al. (1958) Melamed and Green (1963)

Avidin continued

Affinity chromatography using Sepharose	Cuatrecasas and Wilchek (1968)
CMC chromatography followed by crystallization	Green and Toms (1970)

Ovoflavoprotein

DEAE chromatography followed by chromatography	Rhodes et al. (1959) Farrell et al. (1969)

Ovomucoid

Trichloroacetic acid (TCA) precipitation of egg white proteins at pH3.5 followed by acetone precipitation of the TCA soluble fraction	Lineweaver and Murray (1947)
Ion exchange chromatography	Rhodes et al. (1960)

Ovoinhibitor

Ion exchange chromatography of TCA precipitated ovomucoid	Tomimatsu et al. (1966) Liu et al. (1971)
$(NH_4)_2SO_4$ fractionation followed by gel filtration and DEAE chromatography	Davis et al. (1969)

Ficin and Papain inhibitor

$(NH_4)_2SO_4$ fractionation followed by gel filtration and ion exchange chromatography	Fossum and Whitaker (1968)

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The inhibition of vegetative cell outgrowth and division from spores of *Bacillus cereus* T by hen egg albumen

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Spores of *Bacillus cereus* T germinated and formed vegetative cells in Tryptone Soya broth (TSB), pH 9·0 and 7·4 at 30°C. Spores germinated but did not form vegetative cells when suspended in hen egg white (pH 9·0) supplemented with L-alanine and inosine. Using a split image eyepiece, the volumes of germinating spores in egg white were seen to increase as a result of increases in both length and breadth. In TSB at the same pH, the major volume increase resulted from a progressive increase in cell length. Egg white supplemented with L-alanine and inosine (pH 7·6 30°C) allowed limited outgrowth to occur but the vegetative cells differed in morphology to those in TSB. $Fe(NH_4)_2(SO_4)_2.6H_2O$ overcame the inhibition of outgrowth in egg white at pH 7·8 but not in egg white at pH 9·1. Solutions containing trace elements, growth factors and casamino acids could not replace iron in this respect. Sporulation occurred in egg white only when iron was present.

Avian egg white inhibits the growth of the vegetative cells of Gram negative bacteria (Board 1964; Ayres & Taylor 1956; Seviour & Board 1972; Board & Halls 1973), fungi (Silva & Buckley 1962) and yeasts (Schade & Caroline 1944; Silva & Buckley 1962). The older literature leaves the impression that lysozyme is an important, perhaps the major, component of the egg's antimicrobial defence. This can be attributed to the work of Fleming (1922) who observed lysis of bacteria by egg white. It is notable, however, that he used organisms such as *Micrococcus lysodeikticus* which are easily lysed by this enzyme. As yet there is no evidence (Board 1969) to support the notion that lysozyme plays an important role in protecting table eggs during their storage and distribution. Of the many biologically active proteins of the egg white (Osuga & Feeney 1974) the chelator ovotransferrin is probably of much greater importance (Board & Hornsey 1979) because it deprives micro-organisms of Fe^{3+}, a property that is accentuated by the alkaline nature (pH 9–10) of avian egg whites (Sharp & Whitaker 1927). Thus, Schade & Caroline (1944) overcame the inhibition of bacterial and yeast growth by saturating ovotransferrin in egg white with Fe^{3+}.

The fate of bacterial endospores in egg white has attracted little attention. Indeed, Laschtschenko (1909), who noted the lysis of spores of *Bacillus subtilis*, appears to be the only person to study the fate of endospores in egg albumen.

This communication presents evidence that through chelation of Fe^{3+} by ovotransferrin, egg white influences the swelling of germinating spores and prevents the outgrowth of normal vegetative cells.

Materials and Methods

EGGS

Eggs of the domestic hen were stored at 4°C and used within 2 weeks of laying.

0021-8847/82/0200-0067 $02.00

ALBUMEN

The white of the eggs was harvested aseptically by swabbing the shell with 70% (v/v) ethanol, cracking the shell with a flamed scalpel and collecting the contents in a sterile Petri dish. The whites from several eggs were removed with sterile 10 ml wide-bore pipettes, collected in a sterile screw-capped bottle and mixed by gentle shaking. When required, the pH of the white was reduced by slowly passing a gas mixture (5% CO_2–10% O_2–85% N_2) over the surface of the egg white in a sterile Erlenmeyer flask.

ADDITIONS TO THE ALBUMEN

Germinants

To aid germination of endospores L-alanine and inosine (final concentrations 10 and 1 mmol/l, respectively) were added to egg white.

Trace element solution

This contained (per litre distilled water): NaCl 0·3 g; $(NH_4)_2SO_4$ 0·66 g; $ZnSO_4.7H_2O$ 0·11 mg; $CaSO_4.7H_2O$ 0·11 mg; $MnCl_2.4H_2O$ 0·63 mg; $MgSO_4.7H_2O$ 0·14 mg. One millilitre of this filter-sterilized (0·45 μm; Oxoid Ltd) solution was added to 25 ml of egg white.

Growth factor solution

This contained (mg/l distilled water): *p*-aminobenzoic acid 10·0; folic acid 1·0; thiamine 1·0; cyanocobalamin 1·0; nicotinic acid 1·0; pantothenic acid 1·0; riboflavin 1·0; biotin 1·0. One millilitre of this filter-sterilized solution was added to 25 ml of egg white.

Casamino acid solution

Vitamin-free casamino acids (Difco Ltd) were filter-sterilized and added to egg white to give a final concentration of 10 mg/ml.

Iron solution

A solution of filter-sterilized $Fe(NH_4)_2(SO_4)_2.6H_2O$ was added to give a final concentration of 20 μg iron/ml.

MEDIA

Tryptone Soya broth (TSB) (Oxoid Ltd) was made up at a concentration of 30 g/l in 0·2 mmol/l Tris–HCl buffer (pH 7·4 or 9·0) and sterilized at 121°C/15 min.

PRODUCTION AND CLEANING OF SPORES

Bacillus cereus T was grown at 30°C on Potato Glucose Yeast Extract agar (pH 7·2) which contained (% w/v): potato extract (Difco) 0·4; glucose 0·25; yeast extract (Difco) 0·4, in enamel trays. When sporulation was complete and the sporangia had lysed, the spores were washed off the agar with ice-cold distilled water, washed a further six times and harvested by repeated centrifugation (16 000g). Suspensions were cleaned of vegetative cells and debris by discarding the uppermost layers of the pellets obtained by centrifugation. The clean spore suspensions were stored at −20°C.

EXPERIMENTAL TECHNIQUE

To ensure rapid germination, spore suspensions were activated by heating at 70°C/30 min before inoculation into egg white and TSB, to give a final concentration of 10^6 spores/ml. Portions (25 ml) of TSB and egg white (supplemented with L-alanine and inosine) were incubated with gentle shaking (60 shakes/min) in Erlenmeyer flasks in a water-bath at 30°C. Immediately and at regular intervals after inoculation and mixing, a 1 ml sample was removed and mixed with 4 ml of saline–formaldehyde (1·5%–4% w/v) to arrest further spore germination and outgrowth.

MICROSCOPY

Germination

A drop of the spore–saline–formaldehyde suspension was placed on a clean microscope slide and viewed by phase-contrast microscopy. Three hundred spores, present in ten randomly selected fields of view, were scored as phase bright or phase dark and the ratio of germinated (dark) to ungerminated (bright) spores expressed as a percentage.

Length and breadth of the spores

A vernier scale image-splitting eyepiece (Vickers Instruments Ltd) was used and the lengths and breadths of 100 spores in five fields of view measured.

Outgrowth

There are five stages in the outgrowth of vegetative cells from spores of *B. cereus* T (see Fig. 3). The relative percentages of these stages in the population were determined by counting 200 of the spore/vegetative forms.

Morphology

Vegetative cells that had outgrown from spores were examined for shape, size, presence of spores, lipid granules (Burdon 1946) and Gram reaction.

STATISTICAL ANALYSIS

The mean length and breadth of the spores in egg white and TSB (both at pH 9·0 and 30°C) were calculated from 100 samples, in each case the standard deviations were determined and the lengths and breadths in the two media compared using Student's *t* test.

Results

Eighty per cent of the spores suspended in TSB (pH 9·0) and hen egg white (pH 9·0), supplemented with L-alanine and inosine, germinated within 20 min. The rate of germination was the same in both media.

SWELLING

The increases in the lengths and breadths of spores germinating in egg white (pH 9·0), supplemented with L-alanine and inosine, were greater than those in TSB at the same pH (Table 1; Figs 1a & b). With the latter, the breadth of the spores did not increase significantly after 45 min but the length increased markedly (Table 1) until cell outgrowth occurred. In egg white, however, there was an increase in both length and breadth from 60 min onwards as the germinating spore entered a second phase of swelling resulting in a 'balloon-like' form. Both vegetative cell formation in TSB and the second phase of swelling in egg white occurred at approximately the same time indicating that, in egg white, growth continued but outgrowth was inhibited. The exact cause of this swelling is unknown but apart from a small percentage (about 2%) none of these swollen forms proceeded to vegetative cell formation.

Changes in the volume of spores in TSB (pH 9·0) and egg white (pH 9·0) supplemented with L-alanine and inosine are shown in Fig. 2. The volume of the spores in the latter continued to increase for about 4 h but no free vegetative cells were seen. Vegetative cell outgrowth from the spores in TSB occurred after about 70–80 min.

Table 1. Lengths and breadths of *Bacillus cereus* T spores in Tryptone Soya broth and hen egg white (pH 9·0)

Time (min)	Tryptone Soya broth	Egg white	*t* value
0	ML† 0·6818 ± 0·1290	0·6606 ± 0·0942	−1·3223*
	MB 0·4359 ± 0·0795	0·4097 ± 0·0677	−2·5270**
10	ML 0·8069 ± 0·1010	0·8764 ± 0·1420	3·9850***
	MB 0·5688 ± 0·0683	0·6137 ± 0·0621	3·4510***
20	ML 0·8204 ± 0·1230	0·9594 ± 0·1440	7·3434***
	MB 0·6044 ± 0·0744	0·6681 ± 0·0635	6·5085***
30	ML 0·8819 ± 0·1290	0·9673 ± 0·1320	4·6240***
	MB 0·6547 ± 0·0718	0·6827 ± 0·0620	2·9610***
45	ML 0·9448 ± 0·1740	0·9898 ± 0·1360	2·0409****
	MB 0·6573 ± 0·0807	0·7023 ± 0·0623	4·4196***
60	ML 1·0927 ± 0·1950	1·1679 ± 0·1950	2·7269**
	MB 0·6797 ± 0·0619	0·7854 ± 0·0805	10·4190***

† ML = mean length; MB = mean breadth; both in μm.
* $P < 0·10$; ** $P < 0·005$; *** $P < 0·0005$; **** $P < 0·01$; $n = 100$.

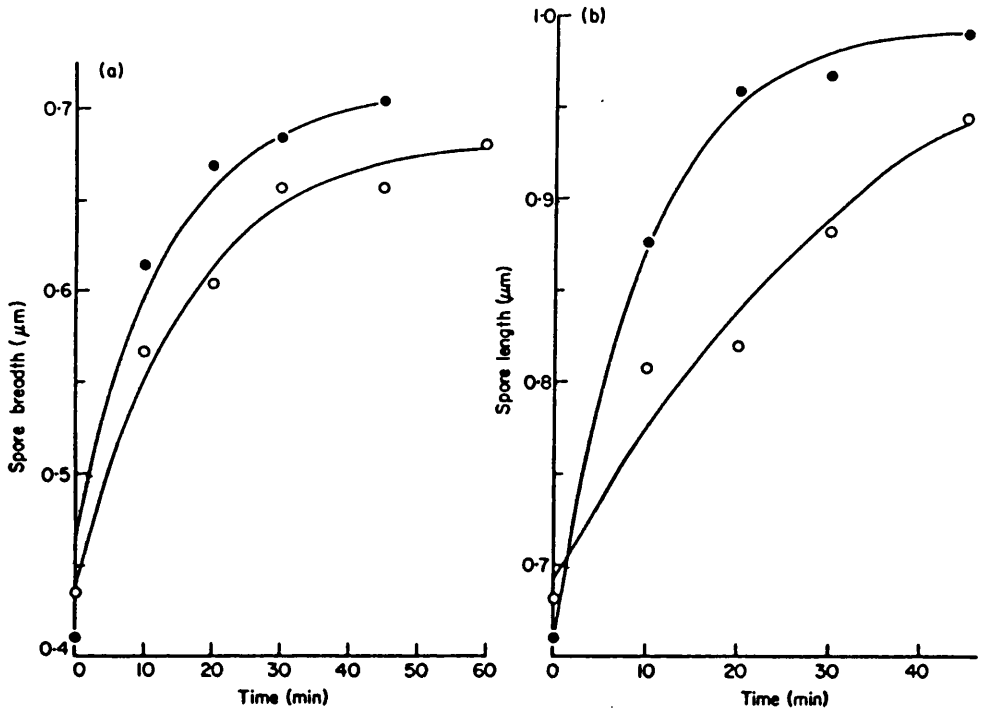

Fig. 1. Increases in (a) breadth and (b) length of spores suspended in ●, egg white; and ○, Tryptone Soya broth (both at pH 9·0). The curves were fitted from the equation: $y - B = A(1 - e^{-Kt})$, where $A = y$ value as t tends to ∞ and $B = y$ at $t = 0$.

VEGETATIVE CELL OUTGROWTH

The germinated spores of *B. cereus* T in TSB passed through five stages (Fig. 3) resulting in free vegetative cells. Samples taken after 60 min in TSB (pH 7·4 and 9·0) showed a progressive shift from the fully germinated spore (stage a, see Fig. 3), to the free vegetative form (stage e).

There was very little vegetative cell formation in egg white (pH 9·0 and 7·6) supplemented with L-alanine and inosine, even after 3 h incubation (Fig. 3). The cells appeared structurally weak and they were always associated with the spore coat. If left overnight, however, some of the spores in egg white (pH 7·6) formed free vegetative cells but these differed from those in TSB in that the

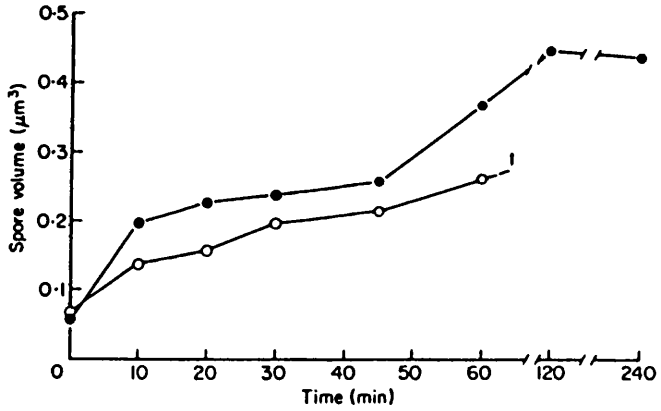

Fig. 2. Increase in volume of spores suspended in ●, egg white; and ○, Tryptone Soya broth (both at pH 9·0). The volume of the spores was calculated on the assumption that the geometry of a spore is a prolate spheroid. † Outgrowth occurred.

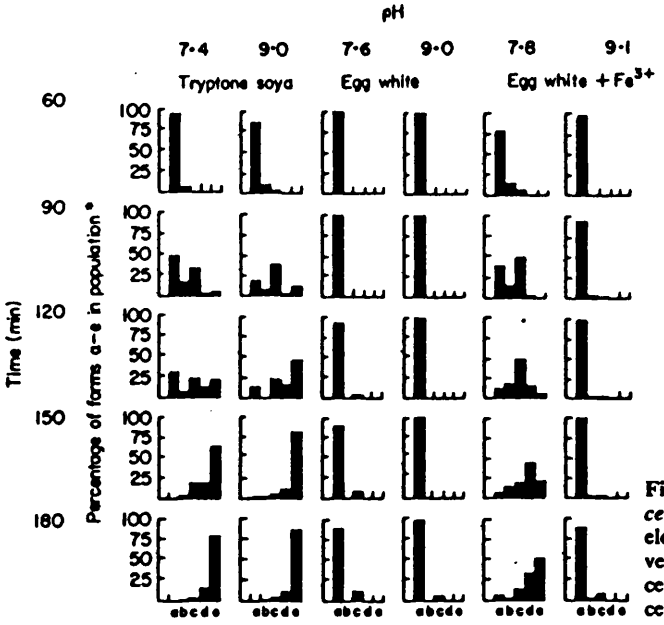

Fig. 3. Stages in the outgrowth* of *Bacillus cereus* T from spores. (a) swollen spore; (b) elongated swollen spore; (c) emerging vegetative cell; (d) emerging vegetative cells showing division; (e) free vegetative cells.

size and shape were irregular and there was no sign of spore formation unlike those from spores in TSB. The cells retained their Gram reaction and contained a small amount of lipid in granule form. Casamino acids, growth factors or trace elements added to egg white (pH 7·6) supplemented with L-alanine and inosine had no effect on the inhibition of outgrowth, however, at this pH iron removed the inhibition and allowed extensive outgrowth to take place. The cells formed in this case had a similar morphology to those that emerged from spores in a rich medium such as TSB. The cells were similar in size and shape but appeared very granular due to the presence of large amounts of lipid. They stained Gram positive and many of the cells contained spores when left overnight. Iron had no effect on the inhibition of outgrowth by egg white (pH 9·1) supplemented with L-alanine and inosine.

Discussion

In most biological fluids near pH 7 and in equilibrium with atmospheric oxygen, Fe^{2+} will be oxidized to Fe^{3+} which is readily hydrolysed to form polynuclear complexes of extremely low solubility (Spiro & Saltman 1969). Such iron is unavailable to micro-organisms unless they are able to solubilize the element from these polymers by the formation of powerful chelates, the siderophores (Snow 1970; Neilands 1972; Lankford 1973). The synthesis of these chelates is enhanced by iron-deficient conditions and in most cases they are excreted into the medium where they complex and solubilize iron present before being taken back into the cell by specific transport systems (Cox *et al.* 1970; Langman *et al.* 1972; Kadner & Bassford 1978).

Avian egg white, by virtue of its content of ovotransferrin is essentially an iron-deficient medium, a condition accentuated by its high alkalinity. In order to grow in this environment, micro-organisms would have to compete with this protein for the iron present. Garibaldi (1970; 1971; 1972) suggested that microbial iron-transport compounds play a significant role in reversing the bacteriostatic action of ovotransferrin and that the synthesis of these compounds is affected at higher growth temperatures.

Oram & Reiter (1968) showed that lactoferrin, the iron-binding protein of milk, which is similar in its action to ovotransferrin, inhibited the outgrowth from spores of *B. stearothermophilus* and *B. subtilis*. These observations confirmed the earlier work of Busta (1966) and Ashton & Busta (1967). Both sets of workers showed that iron could overcome the inhibition. The majority of their work was done using nutrient agar in Petri dishes seeded with the spores and inhibitor added to a well in the agar or in a paper disc. Thus, the conditions differed from those of spores suspended in egg white because of the differences in

the diffusion and nutritional characteristics of the two systems.

It has been shown in this study that of the three main stages in *B. cereus* T spore development—germination; swelling; outgrowth (Hitchins *et al.* 1963)—only the last two stages are affected by hen egg white. The second phase of swelling that occurs with spores suspended in egg white (pH 9·0) supplemented with L-alanine and inosine is possibly due to the synthesis of vegetative cell material; however, because outgrowth is inhibited the spore increases in volume as a consequence. It is also noteable that a marked swelling sometimes occurs when outgrowth is inhibited by other chelating agents such as ethyl picolinate (Pandey & Solanki 1980) and polyphosphates (Gould 1964).

In the same way that saturation of ovotransferrin with iron relieves the inhibition of bacterial growth in egg white (Garibaldi 1960; Board 1964), so iron relieves the inhibition of vegetative outgrowth from *B. cereus* T spores, but only when the pH of the egg white is lowered from 9·0 to 7·9. It would appear then that both the high pH of the egg white and its iron-deficient state are responsible for inhibition of outgrowth. Any vegetative cells that emerge from spores in egg white (pH 7·6) supplemented with L-alanine and inosine appear to have problems such as unequal division and growth which are overcome by the addition of iron so that although the exact mechanism of inhibition is unknown, iron does appear to be involved at some stage during outgrowth.

As growth proceeds the physical and chemical requirements become identical to those for normal vegetative growth. Just if, and how soon, the vegetative form can synthesize iron-transport compounds is unknown. There is no evidence that these compounds are present in bacterial endospores; thus, it is probably the poor capacity for obtaining iron that interferes with the further development of the swollen and newly emergent cells. It was incidentally noticed that spore formation within vegetative cells in egg white occurred only when iron was present suggesting a possible requirement for this ion for sporulation.

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